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Extraction and epitopic analysis of the 53 kd envelope glycoprotein of bovine viral diarrhea virus

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GENERAL INTRODUCTION

Bovine viral diarrhea virus (BVDV) causes several disease processes that are of economic importance in cattle. The virus is found in most cattle populations throughout the world. Serological surveys indicate that 60 to 90 percent of cattle over one year of age have neutralizing antibodies against BVDV. Viral infection of seronegative, immunocompetent, nonpregnant cattle usually results in subclinical disease, followed by the development of viral-neutralizing antibodies. However, infection may also cause reproductive failure, and immunosuppression, and is often associated with respiratory disease complex of calves in feedlots. Infection of pregnant cattle may result in fetal resorption, abortion, fetal mummification, congenital defects, congenital persistent infection and congenital viral specific immunotolerance. A final outcome of congenital persistent infection may be either mucosal disease or chronic BVD, both of which have a high rate of mortality.

BVDV in the United States was first described by Olafson et al. in 1946 (Olafson et al., 1946; Olafson and Rickard, 1947). The bovine viral diarrhea virus agent was first isolated from a bovine cell culture by Lee and Gillespie (1957). Originally, it was thought that two similar but different diseases, bovine viral diarrhea (BVD) and mucosal disease (MD), occurred in cattle. Bovine viral diarrhea is a disease of high morbidity (80-100%) and low mortality (0-20%), whereas mucosal disease has a low morbidity (5-10%) and high mortality (90-100%). In the early 1960's, Pritchard et al., 1961, demonstrated that both diseases were caused by BVDV.

Studies of the molecular characteristics, as well as proteins coded by the virus, have been hampered by the low titers to which BVDV grows in cell culture. Once propagated, the virus is difficult to purify from host cell contaminants. Reports describing BVDV polypeptides have been published (Purchio et al., 1984; Donis and Dubovi, 1987a,b), but a concise and detailed topographic map of relevant neutralizing epitopes has not been reported.

This may be due to the difficulties in purifying virion proteins and the subsequent lack of reliable solid-phase assays. However, a topographic map, based on available monoclonal antibodies, would provide important information about the position of neutralizing epitopes on the viral proteins. Used in conjunction with recent advances in sequencing of the genome of BVDV, an epitopic map could lead to the development of vaccines with greater efficacy. Also, the distribution of specific epitopes within various isolates of BVDV may give a clearer understanding of how the antigenic nature of the virus is altered by mutation.

Explanation of Dissertation Format

The objectives of the research presented here were to: i) develop a solid-phase assay that could be used to detect reactions of antibody with BVDV antigen, ii) generate a topographic map of neutralizing epitopes on the envelope glycoprotein of BVDV, iii) develop a rapid and efficient method for extraction of BVDV antigen, and iv) use the extracted BVDV antigen in a modified solid-phase assay to detect antibody against BVDV in fetal bovine sera.

This dissertation is presented in the alternate format including two manuscripts prepared for submission to scientific journals. The first manuscript was written for submission to Archives of Virology. The second manuscript was written for submission to the Journal of Virological Methods.

The format used for literature cited is that of the Journal of General Virology. A literature review precedes the first manuscript. A general summary and discussion follows the last manuscript. The literature cited within each manuscript appears following that manuscript. Literature cited within the general introduction, literature review, and general summary and discussion is presented at the end of the dissertation.

The Ph.D candidate Robert C. Unfer, was the primary investigator for each of these studies.

REVIEW OF LITERATURE

Bovine Viral Diarrhea Virus

Bovine Viral Diarrhea Virus (BVDV) is the type species of the genus Pestivirus. Other members of this genus include hog cholera virus (HCV) of swine, and border disease virus (BDV) of sheep. BVDV commonly infects cattle, but also may infect sheep, goats, swine, and wild ruminants. While currently classified as non-arthropod-borne members of the Togaviridae family (Mathews, 1982; Westaway et al., 1985), accumulating evidence demonstrating fundamental differences at the molecular level of pestiviruses may dictate their reclassification.

Two biotypes of BVDV exist, cytopathic (CP) and noncytopathic (NCP). These are distinguished on the basis of microscopically observable changes (vacuolation and cell rounding) in cell culture monolayers. BVDV was first propagated in cell culture by Lee and Gillespie (1957) using bovine skin-muscle cells. Initial isolates of BVDV were noncytopathic in cell culture. The presence of virus was detected by inoculation of serologically negative calves. Cytopathic BVDV were originally isolated from tissues of cattle by Underdahl et al. (1957) and Gillespie et al. (1960). Immunological cross-reactivity between both biotypes of virus was demonstrated by several investigators (Gillespie et al., 1961; Pritchard, 1963; Thomson and Savan, 1963). Both CP and NCP isolates induce disease in cattle (Jubb and Kennedy, 1985).

Olafson et al. (1946) originally described an acute, infectious, and highly contagious disease of cattle, characterized by gastroenteritis with severe diarrhea, and a low rate of mortality. Olafson and Rickard (1947) termed the syndrome "virus diarrhea" of cattle. The viral etiology of the disease was proven by reproduction of the disease in calves by using filtrates of serum from other infected calves (Baker, 1954). In nature, horizontal

transmission occurs by inhalation or ingestion of viral particles (Blood et al., 1983). Sources of BVDV are clinically ill or apparently healthy, persistently infected cattle that shed virus in infected saliva, oculonasal discharge, urine, and feces (Malmquist, 1968; Heuschele, 1978; Coria and McClurkin, 1978; Steck, 1980; Kahrs, 1981; McClurkin et al., 1985). Virus may be present in semen, uterine secretion, amniotic fluid, and placental tissue (Coria and McClurkin, 1978; Stober, 1984). In addition, vertical transmission from dam to fetus can occur transplacentally (Romvary, 1965; Malmquist, 1968; Kendrick, 1971). Indirect transmission occurs via fomites, such as feed and equipment (Kahrs, 1981; Stober, 1984).

Acute Infection

Serologic surveys indicate that 60 to 90 percent of all cattle have serum-neutralizing antibodies to BVDV. Clinical disease is seldom observed, indicating that most infections with BVDV are subclinical (Harkness et al., 1978; Stott et al., 1980; Bolin et al., 1985d). Transient pyrexia, leukopenia (Pritchard et al., 1956; Carlson et al., 1957), and viremia (Tyler and Ramsey, 1965) appear four to seven days postinfection, and can last for up to fifteen days (Nuttall et al., 1980; Bolin et al., 1985b). Transient diarrhea may develop in some cattle. Dairy cows often experience a decrease in milk yield. Usually, neutralizing antibodies specific for the virus are produced within two to three weeks after infection and the virus is cleared from the body. Despite apparent life-long antibody production, some cattle may be susceptible to reinfection (Brownlie et al., 1987).

Although most BVDV infections of cattle result in subclinical or mild disease, the immunosuppressive effects associated with infection may enhance the pathogenicity of other agents, such as coronavirus, infectious bovine rhinotraceitis virus (IBR), parainfluenza virus type 3 (PI-3), rotavirus, Actinomyces pyogenes, coccidia, Pasteurella spp., and Salmonella spp. (Reggiardo and Kaeberle, 1981; Yates, 1982; Potgeiter et al., 1984; Edwards et al.,

1986; Baker, 1987). Suppression of interferon production (Diderholm and Dinter, 1966), decreased lymphocyte numbers (Bolin et al., 1985c), impaired lymphocyte function (Muscoplat et al., 1973a,b; Johnson and Muscoplat, 1973; Roth et al., 1986), monocyte chemotaxis (Johnson and Muscoplat, 1973; Ketelson et al., 1979), humoral antibody production (Atluro et al., 1979), as well as polymorphonuclear leukocyte function (Roth et al., 1981) have been demonstrated.

Bovine respiratory disease, which is considered to have a multi-component etiology (Lillie, 1974), is often associated with BVDV infection (Woods et al., 1973). Clinical manifestations of BVDV infection of the respiratory tract have been described by several investigators (Pritchard et al., 1954; Avellini et al., 1968) and include: different types of coughs, an increased respiratory rate, and nasal discharge. BVDV is often isolated with other viruses during outbreaks of respiratory disease. Dual infections of BVDV with PI-3 virus (Nystedt, 1960), and IBR virus (Gratzek et al., 1966) have been reported. Tyler and Ramsey (1965) and Potgeiter et al. (1984) reported that the combination of BVDV and IBR virus caused a more serious infection than either virus alone. The immuno-suppressive effects of BVDV infection may contribute to the pathogenesis of bovine respiratory disease (Malmquist, 1968).

Congenital Infections

Transplacental infection is a frequent sequela to acute infection in the pregnant dam (Binkhorst et al., 1983; Roeder and Harkness, 1986). The gestational age of the fetus determines the outcome of infection (Orban et al., 1983; Liess et al., 1984; McClurkin et al., 1984). For example, fetal infection with either cytopathic or noncytopathic BVDV between 50 and 100 days of gestation may result in fetal resorption, abortion, and mummification (Casaro et al., 1971; Kendrick, 1971; Done et al., 1980). Infection from 100 to 180 days of

gestation may lead to congenital malformations of newborn calves, cerebellar hypoplasia (Brown et al., 1974), ocular abnormalities (Brownlie et al., 1975), or the birth of weak, undersized calves (Done et al., 1980). Infections during the later stages of pregnancy (after 180 days of gestation) may result in the birth of normal calves with antibodies against BVDV.

Persistent Infection

Fetal infection with a noncytopathic viral isolate before 125 days of gestation may result in a congenital, persistent infection with BVDV that is lifelong. Persistently infected cattle are immunotolerant to the virus that infects them, but respond immunologically to a heterologous BVDV (Steck et al., 1980; Liess et al., 1983; Bolin et al., 1985b). Persistently infected calves may be smaller than healthy calves at birth and have a slower rate of growth. The death rate of persistently infected calves may be as high as 50% in the first year of life. Persistently infected calves may be predisposed to infection by microorganisms leading to enteritis and pneumonia (Barber et al., 1985). In addition, persistently infected cattle are at risk for development of mucosal disease. Persistently infected cattle constantly shed BVDV into the environment and are a source of virus for transmission to susceptible animals (Roeder and Harkness, 1986). Persistent infection appears to be an important mechanism by which the virus is maintained in the cattle population. Offspring of persistently infected females are themselves persistently infected (McClurkin et al., 1979; Littlejohns and Walker, 1985).

The prevalence of persistently infected cattle is not precisely known. Up to 1.7% of cattle in the United States may be carriers (Bolin et al., 1985d). Lower rates have been reported elsewhere in the world (Meyling, 1984; Howard et al., 1986; Peters et al., 1987).

Venereal Transmission

Semen from acutely-infected bulls may be contaminated with infectious virus (Whitmore et al., 1978). Semen from persistently-infected bulls contains virus infective to susceptible cows (Coria and McClurkin, 1978; McClurkin et al., 1979; Barlow et al., 1986). Infected semen may be characterized by decreased motility and morphologic abnormalities (McClurkin et al., 1979; Revell et al., 1988). Seronegative dams demonstrate a reduced conception rate until they develop a specific immune response to BVDV. Early embryo loss and repeat breeding difficulties may result from venereal infection (McClurkin et al., 1979).

Mucosal Disease

Ramsey and Chivers (1953) and Ramsey (1954) reported a disease syndrome which was termed "mucosal disease" (MD) due to lesions found on mucosal membranes. Mucosal disease differed from virus diarrhea described by Pritchard et al. (1956) in severity of clinical signs. Usually, less than five percent of a herd presents the symptoms of mucosal disease, but up to one hundred percent of affected cattle may die. Gillespie and Baker (1959) demonstrated that viral isolates from both "virus diarrhea" and mucosal disease conferred mutual protection. Gillespie et al. (1961) demonstrated that the virus isolated from cattle that had "virus diarrhea" and mucosal disease were antigenically related.

Mucosal disease is a sporadic form of BVDV infection of cattle. The disease develops following superinfection of persistently infected cattle with a cytopathic isolate of BVDV (Brownlie et al., 1984; Bolin et al., 1985a). Only certain complementary pairs of cytopathic and noncytopathic BVDV induce mucosal disease (Bolin et al., 1985b). Clinical signs include depression, anorexia, salivation, and elevated temperatures. Cattle often develop a profuse and watery diarrhea, containing mucus and blood. Dehydration and loss of body weight follows. Lesions observed on postmortem examination include: erosions and

ulcers of the esophagus, jejunum, ileum and rectum; elongated ulcers over ileal Peyer's patches, and epithelial necrosis and sloughing in the alimentary tract.

Some cattle develop a chronic wasting disease termed chronic BVD instead of mucosal disease. Clinical signs include inappetance, weight loss, progressive emaciation, and continual or intermittent diarrhea (Pritchard et al., 1956; Johnson and Muscoplat, 1973; Bolin et al., 1987). Lesions of chronic mucosal disease are less severe than those of acute mucosal disease (Muscoplat et al., 1973; Bolin et al., 1987). Cattle with chronic mucosal disease have a congenital persistent noncytopathic BVDV infection, but cytopathic BVDV is not always isolated (McClurkin et al., 1985; Bolin et al., 1987). Infected cattle may survive as long as eighteen months before dying from severe debilitation.

Serological Relatedness of Pestiviruses

The serological relatedness of BVDV to HCV was reported by Darbyshire (1960, 1962), and confirmed by Snowden and French (1968) and Liess et al. (1977). Neutralization assays distinguish BVDV from HCV (Sheffy, 1962), but do not distinguish between BVDV and BDV (Vantsis et al., 1976; Laude and Guelfi, 1979). The serologic cross-relatedness of BVDV, and lack of distinct serotypes, is an accepted convention (Carbrey, 1988; Moennig et al., 1988; Cay et al., 1989). However, antigenic differences demonstrated by cross neutralization tests, using polyclonal sera (Aynaud et al., 1974; Neukirch et al., 1980) and monoclonal antibodies (Bolin et al., 1988; Donis et al., 1988; Magar et al., 1988; Corapi et al., 1988; Corapi and Dubovi, 1990), indicate variation among isolates.

Physical-Chemical Characteristics

BVDV is a small, enveloped, single-stranded, positive-sense RNA virus (Diderholm and Dinter, 1966; Purchio et al., 1983). The diameter has been estimated to be from 40 - 60 nm (Kniazeff and Pritchard, 1960; Hermodsson and Dinter, 1962; Hafez et al., 1968; Maess and Retzlo, 1970; Horzinek et al., 1971). BVDV replication is not inhibited by 5-iodo-deoxyuridine (Hermodsson and Dinter, 1962; Ditchfield and Doane, 1964; Castrucci et al., 1967), but BVDV RNA is sensitive to proflavin and acriflavin (Dinter and Diderholm,1971; Diderholm et al., 1973). The virus is sensitive to ether and chloroform (Hermodsson and Dinter, 1962; Gillespie et al., 1963; Castrucci et al., 1967), and is stable at pH 4.0 - 8.0 (Ditchfield and Doane, 1964). Virus infectivity is greatly reduced by incubation at 56 C for one hour. Ultraviolet light and detergents also reduce infectivity (Westaway et al., 1985). Estimates of BVDV buoyant density range from 1.09 g/ml (Parks et al., 1972) to 1.15 g/ml (Zeegers and Horzinek, 1977; Fernelius, 1968).

Virus particles in electron micrographs appear as pleomorphic, mostly spherical, membrane-bound structures (Bielefeldt-Ohmann and Bloch, 1982). The viral core is an isometric, electron-dense structure, approximately 20 - 30 nm in diameter (Horzinek et al., 1971; Chasey and Roeder, 1981; Gray and Nettleton, 1987). Surface projections cannot be distinguished on purified virus or virus particles in cells and tissues (Stott et al., 1974; Bielefeldt-Ohmann and Bloch, 1982). Surface structures may be lost during concentration and purification procedures (Horzinek et al., 1975). Surface subunits, 5 - 8 nm in diameter and devoid of hemagglutinating activity have been described (Smith et al., 1970). The estimated isoelectric points (pI) of most BVDV proteins are high. Only one BVDV protein, p80, has a predicted pI below 7.0 (Collett et al., 1988b).

Viral Growth Characteristics

Viral replication takes place in the cell cytoplasm, closely associated with the endoplasmic reticulum. Budding of viral particles into cytoplasmic vesicles, endoplasmic reticulum modified into tubules (Chasey and Roeder, 1981; Gray and Nettleton, 1987), and autophagosomes or golgi networks (Bielefeldt-Ohmann et al., 1988) has been observed. Budding of viral particles from the plasma membrane has not been observed. Release of viral particles from infected cells occurs either by disintegration of dead cells, or by an exocytotic event (Bielefeldt-Ohmann and Bloch, 1982).

Kinetic growth studies of infected cell cultures indicate that the virus has a lag phase that lasts six to eight hours post infection (PI), followed by a log phase of growth lasting until twelve hours PI (Nuttall, 1980). A putative cellular receptor for BVDV on the surface of cultured bovine cells has been described (Moennig et al., 1989). A monoclonal antibody directed against a bovine cell-surface protein interfered specifically with BVDV infectivity. However, this interference of infection was not complete. This finding suggests that BVDV entry into bovine cells may be mediated by a specific cell receptor or multiple receptors.

BVDV Genome

Molecular characterization of BVDV has been limited by difficulties with propagation and purification of the virus. Subgenomic virus-specific RNA species have not been detected (Purchio et al., 1983; Renard et al., 1985), distinguishing BVDV from Alphaviruses which produce viral genomic (49S) and subgenomic (26S) RNA's in infected cells (Strauss and Strauss, 1977). The cloned genomes of two cytopathic BVDV isolates, Osloss and NADL, measure approximately 12,500 nucleotides in length, corresponding to a molecular mass of 4.3×10^6 daltons (Renard et al., 1985; Collett et al., 1988a). Dale et al. (1987) reported the cloning of a truncated 1149 nucleotide segment of a third cytopathic viral isolate. Sequence

analysis of the larger cloned genomes, using the method of Pustell and Kafatos (1982, 1984), gave a base composition of A 31.7%, G 25.7%, U 22.2%, C 20.4%. There was a 74% identity between BVDV-NADL and BVDV-Osloss (Renard et al., 1987; Collett et al., 1988a).

The genomic organization of BVDV has been described (Collett et al., 1988a). The viral genome contains a single large open-reading frame (ORF) capable of encoding 3988 amino acids or 449 kDa of protein (Collett et al., 1988a). The structure of the BVDV genome suggests that mature viral proteins are produced by co-translational and post-translational proteolytic processing of a polyprotein precursor.

BVDV RNA represents a minor RNA species in virus-infected cells (Collett et al., 1988a). RNA is isolated from gel electrophoresis as a single, high-molecular weight band that lacks a 3' poly A region. BVDV RNA is refractory to enzymatic poly A addition to the 3' terminus by Escherichia coli polymerase (Renard et al., 1985; Collett et al., 1988a). T4 RNA ligase is able to add cytidine 3', 5'-bisphosphate to the 3' end of BVDV RNA, indicating the presence of a 3' hydroxyl group (Renard et al., 1985). The 5' terminus of BVDV RNA is not labeled with ^{32}p ATP when polynucleotide kinase is used, indicating the presence of a 5' cap (Collett et al., 1988a). Actinomycin D shuts down host cell RNA synthesis, but does not affect BVDV RNA replication (Horzinek, 1981; Purchio et al., 1983), suggesting that the virus carries its own enzyme for RNA synthesis.

BVDV RNA in infected cells appears to possess a high degree of secondary structure, based on observations that BVDV RNA must be denatured to serve as messenger for in vitro reticulocyte translation (Purchio et al., 1984) and exhibits resistance to low concentrations of RNase A (Purchio et al., 1983). The reported sedimentation values of between 24S and 40S, with infectivity sedimenting at the higher values (Moennig, 1971; Purchio et al., 1983); solubility in 2 M LiCl (Collett et al., 1988a); and ability to bind to CF-11 cellulose in the

presence of 15% ethanol (Purchio et al., 1984), are all features found in replicative form RNA. However, sensitivity to higher concentrations of RNase A distinguishes BVDV RNA from true duplex (replicative form) RNA (Purchio et al., 1984). Replicative form or replicative intermediate RNA species of BVDV have not been described, but the secondary structure of BVDV RNA may prevent separation of genomic from replicative form or replicative intermediate RNA species.

BVDV Proteins

Early investigations of BVDV-specific polypeptides varied in the reported numbers and sizes of proteins. Disagreement was probably attributable to the relatively small amount of viral protein found in infected cells, and to the use of different analytical procedures. Pritchett and Zee (1975) detected four ^{3}H-labeled BVDV proteins in infected cells. Coria et al. (1983) detected four major proteins, two of which were glycoproteins, by using coomassie-stained and dansyl-stained polyacrylamide gels. Purchio et al. (1984) found three major and two minor BVDV-specific proteins in virus-infected cell lysates by using radioimmuno-precipitation with convalescent bovine sera. Pocock et al. (1987) used gnotobiotic calf antisera to detect eight BVDV-specific polypeptides in cell lysates by radioimmuno-precipitation. Donis and Dubovi (1987a, b) radiolabeled viral polypeptides in the presence of hypertonic translation initiation blockage, and immunoprecipitated twelve BVDV-specific proteins. Matthaeus (1979) observed the coprecipitation of host cell polypeptides with BVDV polypeptides, and described problems associated with the inefficient inhibition of host cell mRNA and protein synthesis by BVDV.

Collett et al. (1988b) described the cloning of short segments of the BVDV genome as B-galactosidase fusion polypeptides in Escherichia coli. Sequence-specific antisera to these BVDV fusion proteins identified eleven BVDV proteins by immunoprecipitation. This

method accounted for 83% of the coding capacity of the virus genome. The order of BVDV encoded proteins on the genome was also determined. The genome order starting at the 5' end is: p20-gp116-p125-p38-p133. Several precursor-product relationships were suggested by the data. The precise positioning of BVDV proteins within the amino acid sequence is not yet known, however, the viral structural proteins appear to be clustered near the 5' end of the genome.

The first protein of the ORF is a 20 kDa molecule rich in proline residues (11%). The estimated isoelectric point (pI) of this polypeptide is 10.6, and this is the highest of all BVDV proteins (Collett et al., 1988b). A glycoprotein region rich in cysteine residues (3.9%) follows. Viral glycoproteins were identified by immunoprecipitation of [^{35}S]methionine-labeled BVDV-infected cell lysates (Collett et al., 1988b). The putative precursor, gp116, is processed to yield gp62, and gp53. Gp62 is further cleaved to give gp48 and gp25. Fourteen potential N-linked glycosylation sites (Asn-x-Ser/Thr) have been identified in the gp116 sequence (Collett et al., 1988b). Carbohydrate moieties on glycoproteins have been shown to have substantially different molecular weights, ranging from 512 daltons (Graham and Gottscholk, 1960) to 3400 daltons (Spiro, 1962). Variations in reported sizes of BVDV glycoproteins may be due to variations in amounts of bound sugar (Donis and Dubovi, 1987b; Collett et al., 1988b). Partial or complete removal of carbohydrate by treatment with endoglycosidase increased the electrophoretic mobilities of the glycoproteins. Following enzymatic removal of N-linked glycans, gp53 had an estimated size of 42 kd (Collett et al., 1988b). Tunicamycin blocks the addition of N-acetylglucosamine in the synthesis of N-linked glycoproteins. Inhibition of assembly and maturation of other viruses following tunicamycin treatment has been reported (Leavitt et al., 1977). Virus infectivity can be affected following removal of carbohydrate moieties that may be involved in receptor recognition or virus entry (Pizer et al., 1980; Sabara et al., 1982). The addition of

tunicamycin to infected cells reduces the yield of infectious BVDV (Donis and Dubovi, 1987b).

Gp53 is consistently found in virus preparations and is an important antigen stimulating the bovine immune response to BVDV. Experimental data indicate that gp53 is an envelope glycoprotein. Neutralizing monoclonal antibodies recognize gp53 (Bolin et al., 1988; Donis et al., 1988; Corapi and Dubovi, 1990). The mechanism of neutralization is not known, but may be due to interference with viral attachment to host cell receptors.

The p125/p54-p80 region encodes nonstructural viral proteins. The p125 is present in both cytopathic and noncytopathic biotypes, whereas p80 is only produced in cytopathic BVDV isolates (Akkina, 1982; Donis and Dubovi, 1987b; Pocock et al., 1987). Purchio et al. (1984), using tryptic peptide mapping techniques, determined that p80 was included within p125. The function of the remaining viral proteins, p133 and its cleavage products p75 and p58, has not been determined.

The identity of the BVDV nucleocapsid protein is not known. The p20 protein is a likely candidate for the nucleocapsid protein because it has a high isoelectric point (10.6), a high proline content, and is encoded within the genomic region suspected to include the viral structural proteins (Collett et al., 1988b).

Antigenic Analysis

Epitopes of proteins can be divided into two categories; sequence-dependent and conformationally-dependent (Sela, 1969). Sequence-dependent epitopes are made up of a single, continuous length of the amino acid primary sequence. Conformationally-dependent epitopes are comprised of amino acids that may be widely separated in the primary structure, but are brought in close proximity on the surface of the protein when the polypeptide chain is folded to form the structure of the native protein.

The term epitope has been applied to surface configurations, single determinants, immunogenic elements, haptenic groups, and antigenic patterns (Jerne 1960). Extrapolation studies with carbohydrate antigens have fostered the idea that protein epitopes are formed by hexapeptides. Current evidence indicates that epitopes on native proteins occupy large areas comprised of 15 - 22 amino acid residues in discontinuous array (Padlan et al., 1989; Tulip et al., 1989).

Since their original description (Kohler and Milstein, 1975), monoclonal antibodies have been invaluable tools for investigating the antigenic relationships between viruses and viral proteins. The basic property of monoclonal antibodies, their recognition and specific binding to one epitope or family of related epitopes, may be used to provide information on structure, function, relatedness, synthesis and processing, and/or distribution. As illustrated by antigenic analysis performed with rabies virus (Flammand et al., 1980), minor differences between closely related proteins undetected by polyclonal sera may be readily evident by analyses with monoclonal antibodies. These minor antigenic differences, relevant in virus-host interactions, are important in the development of vaccine programs for diseases caused by antigenically unstable viruses.

Monoclonal antibodies may be tested for their cross reactivity with a panel of test antigens (viral isolates). The binding of antibodies in distinct reactivity patterns to a vital protein indicates the presence of distinct (operationally defined) epitopes on that protein (Yewdell and Gerhard, 1981). However, the topographic relationship between individual epitopes remains undefined. Three experimental approaches may be used to dissect the antigenic topology. First, the capacity of monoclonal antibodies to compete with each other for their individual epitopes on the viral protein can be determined. Second, the structural properties of the epitopes themselves can be assessed. Third, individual epitopes can be

grouped and arranged relative to each other by comparative analysis of antigenic variants selected for in the presence of monoclonal antibodies (Yewdell and Gerhard, 1981).

Monoclonal antibodies were first used for antigenic analysis of togaviruses by Roehrig et al. (1980). Their application paralleled methods first introduced in studies with influenza virus (Webster and Laver, 1980) and murine ecotropic leukemia virus (Lostrum et al., 1979). The spatial orientation of epitopes recognized by a panel of monoclonal antibodies with respect to one another is defined in a competitive binding assay. Antibodies that mutually block each others binding recognize epitopes that are identical or closely associated spatially. Conversely, Heinz et al., (1984) working with several flaviviruses, demonstrated enhancement of binding by monoclonal antibodies recognizing spatially distant epitopes.

Antigenic analysis of the immunologically important E2 glycoprotein of Venezuelan Equine Encephalitis (VEE) virus resulted in the identification of eight epitopes (Roehrig et al., 1982; Roehrig and Mathews, 1985). Similar studies with Semliki Forest virus (SFV) demonstrated the presence of five epitopes on the E2 glycoprotein (Boere et al., 1984). While polyclonal neutralizing antibodies are not produced against the togavirus E1 glycoprotein, analysis of VEE E1 glycoprotein with monoclonal antibodies identified four epitopes, one of which neutralized virus infectivity. The explanation for viral neutralization by a monoclonal antibody against the E1 glycoprotein was provided by the deduced spatial orientation of identified epitopes. The E1-neutralizing epitope was in close proximity to the E2 glycoprotein in the togavirus E1-E2 glycoprotein dimer, indicating that steric hindrance of cell binding sites on the E2 occurred following binding of the monoclonal antibody to E1. Sindbis virus and SFV were shown to have five and six epitopes respectively (Schmaljohn et al., 1983; Boere et al., 1984). Competitive binding assays with Western Equine

Encephalitis virus (WEE) revealed eight epitopes existed on the E1 glycoprotein (Hunt and Roehrig, 1985)

A topographical model of epitopes on the structural glycoprotein E, for several flaviviruses, has been constructed. The most extensive analysis was performed with Tick-Borne Encephalitis (TBE) virus. Guirakhoo et al. (1989) identified nineteen epitopes clustered in three non-overlapping antigenic domains. Reactivities of monoclonal antibodies and their affects upon functional activities associated with the E glycoprotein (Hemagglutination-inhibition, neutralization, passive protection) were different (Heinz et al., 1983). Competitive binding assays have also been performed with Japanese Encephalitis (JE) virus (Kimura-Kuroda and Yasui, 1983,1984; Cecilia et al., 1988), Yellow Fever (YF) virus (Schlesinger et al., 1984; Cammack and Gould, 1986), and Dengue viruses (Henchal et al., 1985).

Diagnostic and Quantitative Assays

The earliest detection method for BVDV was direct inoculation of cattle (Olafson et al., 1946). A serum neutralization test, using animals, was first reported by Baker et al. (1954). This was followed by a serum neutralization test (SNT) in cell culture, using cytopathic BVDV (Gillespie et al., 1960). The phenomenon of interference of cytopathic viral isolates by noncytopathic BVDV provided a means of titration and identification without the necessity of animal inoculation (Gillespie et al., 1962). In cell cultures dually infected with both biotypes of BVDV, noncytopathic virus interferes with the production of CPE by the cytopathic isolate (Gillespie et al., 1962). CPE is characterized by vacuolation, rounding, and detachment of infected cells (Gillespie et al., 1960). Gutekunst and Malmquist (1964) used a modification of the interference test, and developed a neutralization test for noncytopathic BVDV isolates. Concurrently, Inaba et al. (1963) described an assay

for noncytopathic BVDV isolates that took advantage of the ability of BVDV to exalt Newcastle Disease virus (NDV) in cell culture. This procedure was termed the END method, for exaltation of Newcastle Disease Virus.

Kniazeff and Pritchard (1960) reported using a cell culture plaque assay to conduct neutralization tests of various BVDV antisera against the Oregon C24 virus isolate. Later, Gatzek et al. (1967) published a more detailed description of a BVDV plaque assay. Darbyshire (1962), and Gutekunst and Malmquist (1963), used an agar gel diffusion assay to study the serological relationships between BVDV and other pestiviruses.

Mengeling et al. (1963) first described an indirect fluorescent antibody test (IFA) in which conjugated anti-BVDV serum specifically stained bovine cells infected with BVDV. In 1964, Fernelius detected and titrated noncytopathic BVDV with fluorescein-conjugated antibody specific for either cytopathic of noncytopathic BVDV isolates. A complement-fixation test (CF) for BVDV, using soluble antigen prepared from infected cell culture fluids, was reported by Gutekunst and Malmquist (1964).

Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme immunoassays originated with the work of Avrameas and Uriel (1966), who proposed labelling antigens and antibodies with enzymes for use in conventional serological techniques. Viral antigens in animal tissue sections were located by enzyme-labelled antibodies by Nakane and Pierce (1966), and Wincker and Avrameas (1969). Engvall and Perlmann (1971, 1972) coined the term ELISA to describe an assay comparable in sensitivity to radioimmunoassays. Polystyrene tubing was used as an immunosorbent support.

Subsequently, Voller et al. (1974) developed an ELISA by using polystyrene plates, and applied this method to the detection of antibodies to malaria (1974), rubella (1975),

measles, and cytomegalovirus (1976). In 1975, Ruitenberg et al. first applied the ELISA methodology to animal diseases, for the detection of antibodies to Trichinella spiralis. Advances in ELISA technology continued with togaviruses. Gravell et al. (1977) described the detection of antibodies to rubella virus. The detection of antibodies to the alphavirus VEE by ELISA was reported by Frazier and Shope (1979). This was followed by the application of ELISA to the detection of antibodies to flaviviruses: TBE (Hofmann et al., 1979), and dengue virus (Dittmar et al., 1979).

Chu et al. (1985) and Howard et al. (1985) reported the first ELISA for the detection of antibodies to BVDV. Liauw and Eugster, (1986) described an ELISA for the quantitation of immunoglobulin G to BVDV. Additional reports of indirect ELISA (Bock et al., 1986; Caquineau et al., 1986; Chu et al., 1987; Katz and Hanson, 1987) and blocking ELISA (Westenbrink et al., 1986; Katz and Hanson, 1987) for the detection of antibodies to BVDV have been made. Juntti et al. (1987) reported the use of monoclonal antibodies in indirect and competitive blocking ELISA to detect BVDV antibodies, and Justewicz et al. (1987) described a procedure using BVDV-infected cell monolayers for detection and quantitation of BVDV-specific antibodies.

SECTION I: EPITOPIC ANALYSIS OF THE 53KD ENVELOPE GLYCOPROTEIN OF BOVINE VIRAL DIARRHEA VIRUS

Epitopic Analysis of the 53kd Envelope Glycoprotein of Bovine Viral Diarrhea Virus

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ABSTRACT

Thirteen monoclonal antibodies were prepared against five isolates of bovine viral diarrhea virus (BVDV). All of the antibodies reacted with viral protein gp53. In the absence of serum complement, each antibody neutralized infectivity of BVDV. The monoclonal antibodies were used in a competitive binding assay (CBA) to determine the topographic relationship of neutralizing epitopes on gp53. Two distinct antigenic domains on the gp53 of cytopathic and noncytopathic isolates of BVDV were identified. The majority of monoclonal antibodies recognized overlapping epitopes in a single domain. A possible second domain was identified by the binding of one monoclonal antibody. A dot-immunoblot assay using denatured viral protein was performed. The results indicated that the neutralizing epitopes on gp 53 were conformationally-dependent. Endoglycosidase treatment of viral glycoproteins significantly reduced or prevented the binding of all thirteen monoclonal antibodies.

INTRODUCTION

Bovine viral diarrhea virus (BVDV), an enveloped, single-stranded positive-sense RNA virus, is the type species of the Pestivirus genus in the family Togaviridae (Mathews, 1982; Westaway et al., 1985). Other members of the genus include hog cholera virus (HCV), and border disease virus of sheep (Horzinek, 1981), and all are closely related antigenically (Darbyshire, 1960, 1962; Fernelius et al., 1973; Osburn et al., 1973; Liess et al., 1977). BVDV is an economically important pathogen that induces a wide range of clinically mild to fatal diseases of cattle. There are two biotypes of BVDV which are differentiated by their ability to induce cytopathology in bovine cell cultures. Despite detectable biologic and molecular differences between both biotypes, detectable antigenic variation has been difficult to classify using conventional methods (Liess et al., 1977). Recent studies have indicated the potential of monoclonal antibodies (Mabs) to segregate isolates of BVDV into groups (Bolin et al., 1988).

Molecular cloning of the BVDV genome has allowed a preliminary definition of the polypeptides encoded by the virus (Collett et al., 1988a, b). The identity of the BVDV glycoproteins was established by immunoprecipitation, using lysates of BVDV-infected cells labeled with [^{3}H] mannose (Donis and Dubovi, 1987), [^{3}H] glucosamine (Pocock et al., 1987), and [^{35}S] methionine, followed by analysis of endoglycosidase-treated polypeptides (Collett et al., 1988b). The 53 kd glycoprotein of BVDV possesses epitopes to which virus-neutralizing Mabs are directed (Bolin et al., 1988; Corapi et al., 1988; Donis et al., 1988; Magar et al., 1988). Previous studies have used reactivity patterns of large numbers of BVDV isolates with panels of Mabs to examine the antigenic nature and distribution of epitopes on the 53 kd glycoprotein (Bolin et al., 1988; Mateo Rosell, 1988; Moennig et al.,

1989; Kreeft et al., 1990). While those studies indicated the presence of multiple epitopes on gp53, the topographic arrangement of those epitopes was not addressed.

In this report, we describe the construction of topographic maps of epitopes on gp53 of two BVDV isolates using competitive binding assays with radiolabeled Mabs. Furthermore, the dependence of these epitopes upon serum complement for virus neutralization was determined. And a preliminary examination of the position on the glycoprotein relative to carbohydrate moieties was made.

MATERIALS AND METHODS

Monoclonal Antibodies

All monoclonal antibodies used in this study have been previously described (Bolin et al., 1988) except for N2, BZ-2, and BZ-4, which were generated during this study in Dr. Bolin's laboratory. Balb/C or RBF/Dn mice (The Jackson Laboratory, Bar Harbor, ME) were immunized intraperitoneally or intrasplenically with concentrated and partially purified BVDV isolates, and hybridoma cells were produced by fusion of spleenic cells from immunized mice with Fox/NY (Taggart and Samloff, 1983) or SP2/O /Ag14 myeloma cells as described by Peters et al. (1986). Screening for anti-BVDV Mab was performed by virus neutralization and immunohistochemical procedures, including indirect immunofluorescence assay (IFA), and immunoperoxidase assay (IP), (Ridpath et al., 1991). Antibody isotypes were determined by double immunodiffusion test using isotype-specific antisera (Litton Bionetics, Kensington, MD), and Mab specificity was established by immune precipitation of ^{35}S-labeled lysates of bovine turbinate (BT) cells infected with BVDV (Bolin et al., 1988).

Hybridoma cells secreting anti-BVDV neutralizing Mab were grown in serum-free Dulbecco's MEM supplemented with AAT (7.5 x 10^{-5}M adenine, 8 x 10^{-7}M aminopterin and 1.6 x 10^{-5}M thymidine). Cells were propagated either in suspension, or in cell culture flasks containing BT cell feeder layers. Supernatant from each hybridoma cell line was collected and stored at 4 C. Ascitic fluid for each Mab was produced in eight-week-old Balb/C mice primed with 0.5 milliliter of incomplete Freund's adjuvant.

Purification and Labeling of Monoclonal Antibodies

Mabs secreted from hybidomas grown in serum-free medium were precipitated with ammonium sulfate and dialyzed against .001M saline. Mabs were resuspended in phosphate

buffer (0.02M, pH 7.0) and applied to a protein G sepharose column (Pharmacia LKB, Uppsala, Sweden). The Mabs were eluted from the column with 0.1M glycine-HCl (pH 2.7). Fractions of eluate (1 ml) were collected and analyzed for protein content by absorbance at 280 nm UV light (Beckman DU-50 spectrophotometer, Beckman Instruments, Palo Alto CA). Fractions containing Mab were pooled, dialyzed overnight against 0.001M phosphate buffer pH 7.0, and concentrated by lyophilization (Virtis Lyphilizer, Gardiner, NY). The lyophilized Mabs were resuspended in ddH_2O, and protein concentrations were determined. (Bio-Rad Protein Assay, Bio-Rad, Richmond, CA).

Concentrated Mabs were labeled with Na-^{125}I using Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril, Pierce Chemical Co., Rockford, IL) by the method of Fraker and Speck (1978). The labeling reaction was stopped by gel filtration chromatography (Bio-gel P-10, Bio-Rad). One milliliter fractions were collected and their radioactivity quantitated (DP 5500 Gammacounter, Beckman Instruments). Fractions were analyzed by SDS-PAGE in a discontinuous buffer system as described by Laemmli (1970). Gels were dried, and the presence of labeled heavy and light immunoglobulin chains was determined by autoradiography. Fractions containing labeled Mab were stored at -20 C.

Virus, Cells, and BVDV Antigen Preparation

The Singer isolate of cytopathic BVDV and the 7443 isolate of noncytopathic BVDV, were propagated in monolayers of BT cells as described previously (Ridpath et al., 1991). Cells infected with the cytopathic Singer virus were harvested when 90% of cells displayed cytopathic effect (18-22 hr post-infection). Cells infected with the noncytopathic 7443 virus were harvested 48 hr post-infection.

Detergent solubilized antigen was prepared from BVDV-infected and uninfected BT cells scraped off three 150 cm^2 cell culture flasks and solubilized using 13 mM of CHAPS

(3-[(3-cholamidopropyl-dimethyl-ammonio]-1-propanesulfonate), Pierce) in phosphate buffered saline (PBS), pH 7.2 (2.5 ml/flask). Following a one hour incubation on ice with stirring, the mixture was sonicated (15 sec), and cell material was sedimented by centrifugation for 15 minutes at 12,000 x g (Beckman eppendorf centrifuge, Beckman). Detergent was removed by overnight dialysis against 0.001M phosphate buffer, pH 7.0, or by chromatography (Extracti-Gel-D column, Pierce).

Complement-Dependence Assay

Neutralization titers of anti-BVDV Mabs in the presence or absence of serum complement were measured. Complement-depleted fetal bovine sera was prepared by incubating one hour at 65 C before addition to F-15 Eagle's minimum essential media (MEM) (Grand Island Biological Co., GIBCO, Grand Island, NY, Gibco). BVDV-Singer and BVDV-7443 were propagated in complement-depleted MEM. Serial two-fold dilutions of Mabs were mixed 1:1 with virus lysate containing one hundred infectious particles and incubated for one hour at 37 C in 96-well microtiter plates (Costar, Cambridge, MA). Following addition of 10^4 BT cells to each well, microtiter plates were incubated at 37 C for seventy-two hours and examined for viral neutralization using cytopathic effect or immunoperoxidase staining to detect virus.

Antibody Binding Curves and Antibody Binding Constant Determination

The relative avidity for each labeled Mab was determined by coating and preparing polyvinyl chloride (PVC) 96-well microtitration plates (Dynatech Industries, Chantilly VA) with solubilized BVDV antigen or uninfected BT-cell antigen, by the method of Frankel and Gerhard (1979). Antigen coated plates were incubated with blocking buffer (0.5% fish gelatin in PBS) (Saravis, 1984) for one hour at room temperature. Dilutions of labeled Mab

were added to microtitration plate wells and incubated three hours at room temperature. The microtitration plates were then washed six times each with TNE/W (0.5 M Tris-HCl, 0.15 M NaCl, 5 mM EDTA, pH 7.4, 0.5% Tween-20), and ddH_2O. Individual microtitration plate wells were cut out and the amount of bound labeled Mab was quantitated as before. Data were expressed as antigen-binding curves. Straight line regression analysis of the data was used to calculate antibody binding constants of each Mab (Frankel and Gerhard, 1979).

Competitive Binding Assays (CBA)

PVC microtiter plates were coated with solubilized BVDV antigen or uninfected BT cell antigen (0.1 ug/well), and prepared as before. Dilutions of labeled, purified Mabs, and nonlabeled competing Mabs (ascitic fluid) were made in TNE/W containing 0.1% fish gelatin. Labeled Mabs were diluted to the concentration at which 50% binding occurred as demonstrated in antibody binding curves, while nonlabeled competing Mabs (ascites fluid) were diluted ten-fold. Each CBA was performed using pairs of ^{125}I-labeled and nonlabeled Mabs. Simultaneous and consecutive CBA's were performed to distinguish competition that may have been due to disparate antibody affinities. In simultaneous binding assays (one-step), dilutions of labeled and nonlabeled competing Mab were mixed together, added to antigen-coated microtitration plate wells, and incubated three hours at room temperature. Each reaction was performed in triplicate. After incubation, the microtitration plates were washed six times each with TNE/W and ddH_2O, individual wells were cut out, and the amount of bound labeled Mab was quantitated as before. In consecutive binding assays (two-step), dilutions of competing antibody were added to antigen-coated microtitration plate wells and incubated three hours at room temperature. The microtitration plates were then washed six times with TNE/W, labeled Mab was added to each well, and the plates incubated for three hours at room temperature. The microtitration plates were washed six times each

with first TNE/W, and then with ddH_2O. The microtitration plate wells were cut out and the amount of bound labeled Mab was quantitated as above.

Reciprocal assays with pairs of Mabs were performed to identify competition that may have been due to steric hindrance rather than true competition for epitopes. Reduction in binding of the labeled Mab by unlabeled antibody was calculated using the formula:

$$1 - \frac{[(\text{total cpm} - \text{background cpm}) - (\text{cpm} - \text{background cpm})]}{\text{total cpm} - \text{background cpm}} \times 100$$

and expressed in terms of percent competition. Competition between two Mabs for an epitope was indicated by a reduction of 60% or more in binding of the labeled antibody. Reduction of binding by 40 to 60% identified weak competition between pairs of Mabs. An increase of 50% or greater in binding of ^{125}I labeled Mabs identified enhancement of antibody binding.

Endoglycosidase Treatment of Viral Antigen

Detergent solubilized viral and uninfected cell protein were treated with Endo-glycosidase H and F/N-glycosidase F (Boehringer-Mannheim Biochemicals, Indianapolis, IN) as described by Collett et al. (1988b). Enzyme treated and untreated protein mixtures were coated onto PVC microtitration plates wells and prepared as before. Labeled Mabs were added to the microtitration plate wells and incubated for three hours at room temperature. The microtitration plate wells were washed as above, cut out, and the amount of bound labeled Mab was quantitated as described.

RESULTS

Labeling of Monoclonal Antibodies

The procedures used to purify Mabs produced in serum-free media yielded about 50 ug of Mab per 100 ml of cell culture supernatant. Extraneous proteins were not detected by electrophoretic analysis of purified Mabs. Levels of incorporation of the ^{125}I varied among Mabs, regardless of the concentration of antibody used (table 1). Iodination of Mabs CA 34, and CA 80, resulted in low levels of incorporation of radioactivity. Differences in labeling patterns of immunoglobulins were detected by autoradiography. Both heavy and light chains of all Mabs were labelled, except for CA 80 which incorporated label in only the light chain.

Antibody Avidity and Affinity Determination

The relative avidity of each Mab for BVDV antigen was estimated from antigen binding curves in which antibody was titrated against constant amounts of bound antigen. The labeled Mabs were assayed against antigen from cytopathic BVDV-Singer and noncytopathic BVDV-7443 BVDV. Data were expressed in the form of antibody-binding curves that showed binding of each dilution of labeled Mab to bound BVDV antigen. The maximum level of binding (a measure of the relative avidity), and the Mab concentration at which 50% binding occurred, were determined. Figure 1 shows the antigen-binding curves for Mabs titrated against BVDV-Singer and BVDV-7443 antigen. The maximum level of binding (plateau level) varied among Mabs.

Antibody binding constants for each Mab were calculated from regression analysis of data from the antigen-binding curves (table 2). Two Mabs, BZ-2 and BZ-4, had binding constants that were lower than the remainder of the panel by a factor of ten, while CA 36 had

the highest binding constant by a factor of ten. The remainder of the Mabs exhibited similar binding constants.

Competitive Binding Assays (CBA)

Twelve of thirteen Mabs bound epitopes on gp53 of the Singer isolate of virus (table 2). Figure 2 shows the results of simultaneous competitive binding assays for neutralizing epitopes on gp53 of the Singer isolate between unlabeled competing Mabs and ^{125}I-labeled Mabs. Results of consecutive competitive binding assays were nearly identical (data not shown). Table 3 lists the results of each competitive binding assay performed with the Singer isolate of cytopathic BVDV.

Ten of thirteen Mabs bound epitopes on gp53 of noncytopathic BVDV-7443 (table 2). Figure 3 shows the results of simultaneous competitive binding assays for neutralizing epitopes on gp53 of the 7443 isolate between unlabeled competing Mabs and ^{125}I-labeled Mabs. Results of consecutive competitive binding assays were nearly identical (data not shown). Table 4 lists the results of each competitive binding assay performed with the 7443 isolate of noncytopathic BVDV.

Non-reciprocal enhancement of binding by ^{125}I-labeled Mabs was observed in competitive binding assays with the 7443 isolate of noncytopathic BVDV. The binding of Mab CA 39 was enhanced by Mabs CA 34, CA 36, and N2, While the binding of antibodies CA 36, and N2 was enhanced by Mab CA 80.

Potential topographic maps of neutralizing epitopes on gp53 of the cytopathic Singer isolate and the nocytopathic 7443 isolate of BVDV defined by this panel are shown in figure 4. Eleven Mabs reacted with overlapping epitopes in one antigenic domain on gp53 of the Singer isolate. A possible second antigenic domain was defined by CA 78, which did not compete with the other Mab. Nine of the ten Mabs recognized overlapping epitopes in one

antigenic domain on gp53 of the 7443 isolate. CA 24, which did not compete with the other Mabs defined a possible second antigenic domain.

Further Characterization of GP53 Epitopes

The neutralizing epitopes on gp53 were further characterized using a complement-dependence assay, and by antibody binding assays using endoglycosidase treated viral protein. Neither the presence or absence of complement affected viral neutralization by any Mab (table 5). Treatment of viral antigen with endoglycosidase H and F/N-glycosidase F greatly reduced or inhibited the binding of all thirteen Mabs (results not shown).

Table 1. Description of Monoclonal Antibody and Immunoglobulin Subclasses and Specific Activities After Iodination.

Monoclonal Antibody	Fusion Partners	Isotype	Immunogen[3]	Specific Activity
CA 1	RBF/DN[1] - Fox/NY[2]	IgG1	NADL	3.10 uCi/ug
CA 3	RBF/DN - Fox/NY	IgG1	NADL	2.58 uCi/ug
CA 24	RBF/DN - Fox/NY	IgG1	7443	4.22 uCi/ug
CA 34	RBF/DN - Fox/NY	IgG2a	7443	1.14 uCi/ug
CA 36	RBF/DN - Fox/NY	IgG2b	7443	6.30 uCi/ug
CA 39	RBF/DN - Fox/NY	IgG1	Singer	2.69 uCi/ug
CA 72	RBF/DN - Fox/NY	IgG1	Singer	2.38 uCi/ug
CA 78	RBF/DN - Fox/NY	IgG3	Singer	3.01 uCi/ug
CA 80	RBF/DN - Fox/NY	IgG1	Singer	1.35 uCi/ug
CA 82	RBF/DN - Fox/NY	IgG1	Singer	2.23 uci/ug
N2	RBF/DN - Fox/NY	IgG1	Neb-Rk13	3.59 uCi/ug
BZ-2	Balb/C - SP2/O	IgG2b	88055	2.67 uCi/ug
BZ-4	Balb/C - SP2/O	IgG1	88055	3.94 uCi/ug

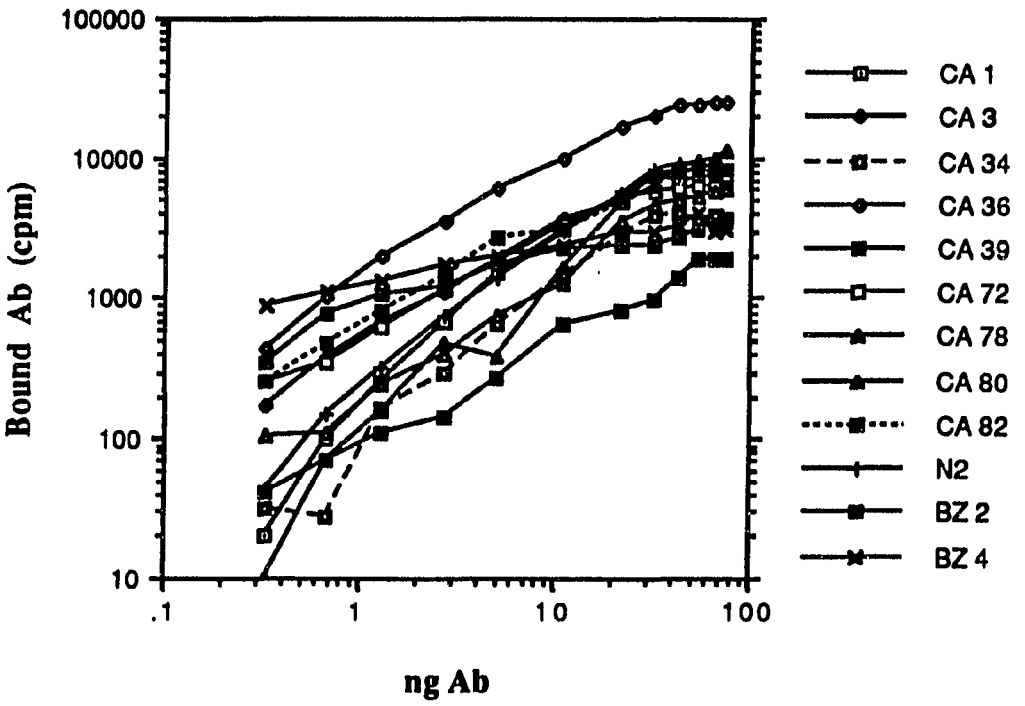
[1] RB/DN mouse strain

[2] Fox/NY myeloma cell line

[3] Cytopathic viruses - Singer, NADL, 88055
Noncytopathic viruses - 7443, Nebraska, Rk13

Figure 1: BVDV-Singer and BVDV-7443 antibody-binding curves. Dilutions of labeled, purified Mab were added to treated antigen coated PVC microtiter wells in triplicate, and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated. Data was use to construct antibody-binding curves to determine relative avidity of each Mab.

Singer-Antibody-Binding Curve



7443-Antibody-Binding Curve

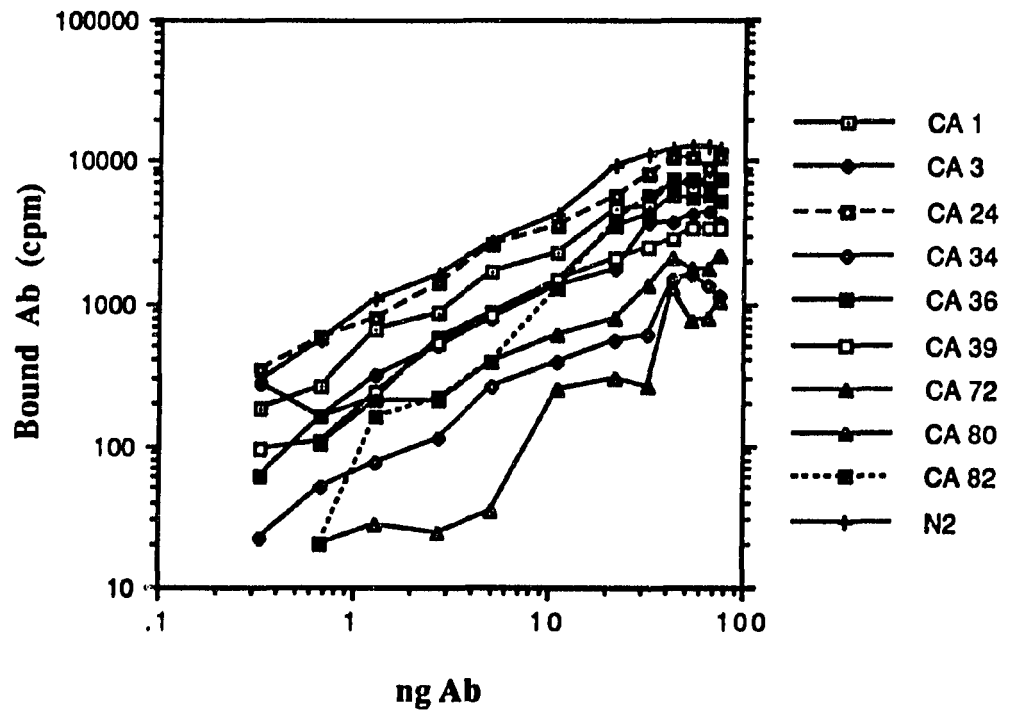


Table 2. Binding Constants of Monoclonal Antibodies specific for BVDV.

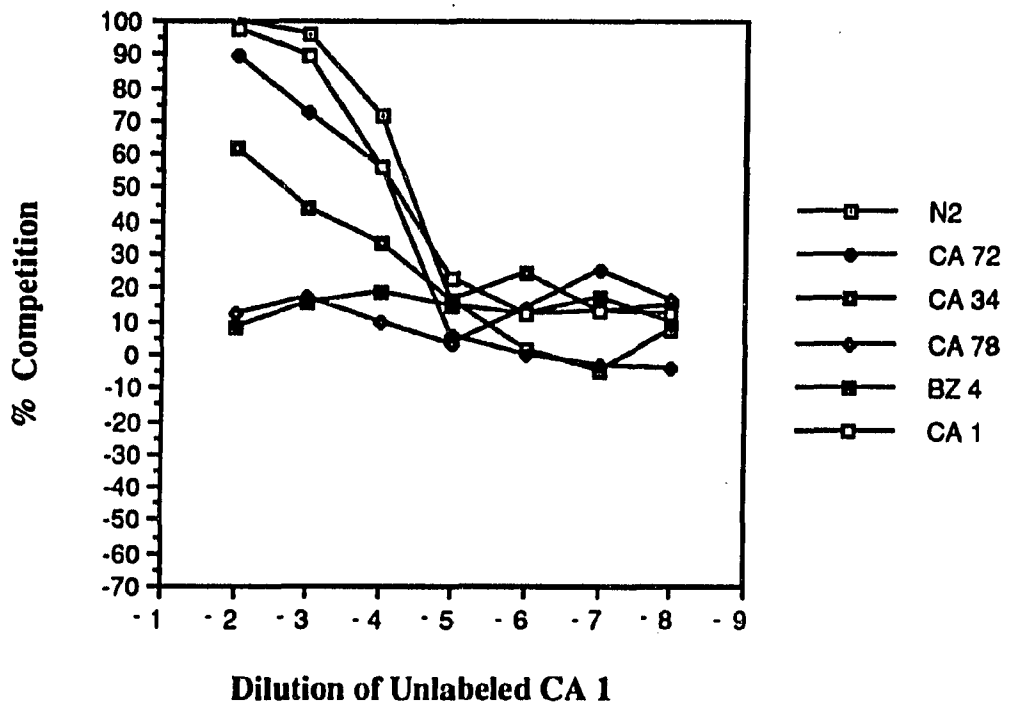
Monoclonal Antibody	Binding Constant[1]	BVDV-Singer	BVDV-7443
CA 1	$2.65 \pm .05 \times 10^8 M^{-1}$	+[2]	+
CA 3	$1.52 \pm .07 \times 10^8 M^{-1}$	+	+
CA 24	$5.57 \pm .02 \times 10^8 M^{-1}$	-[3]	+
CA 34	$5.07 \pm 0.1 \times 10^8 M^{-1}$	+	+
CA 36	$8.30 \pm .02 \times 10^8 M^{-1}$	+	+
CA 39	$3.75 \pm .05 \times 10^8 M^{-1}$	+	+
CA 72	$2.14 \pm .09 \times 10^8 M^{-1}$	+	+
CA 78	$2.60 \pm .07 \times 10^8 M^{-1}$	+	-
CA 80	$2.43 \pm .09 \times 10^8 M^{-1}$	+	+
CA 82	$5.73 \pm .09 \times 10^8 M^{-1}$	+	+
N2	$3.63 \pm .04 \times 10^8 M^{-1}$	+	+
BZ 2	$1.52 \pm .08 \times 10^8 M^{-1}$	+	-
BZ 4	$2.23 \pm .02 \times 10^8 M^{-1}$	+	-

[1] Determined by regression analysis of titration curves with the Singer isolate of cytopathic BVDV for all Mabs except CA 24.

[2] Binds to viral antigen

[3] Does not bind to viral antigen

Figure 2: Simultaneous competitive binding assays of BVBV-Singer using Ca 1 and Ca 78. Dilutions of labeled purified Mab (50% binding concentration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.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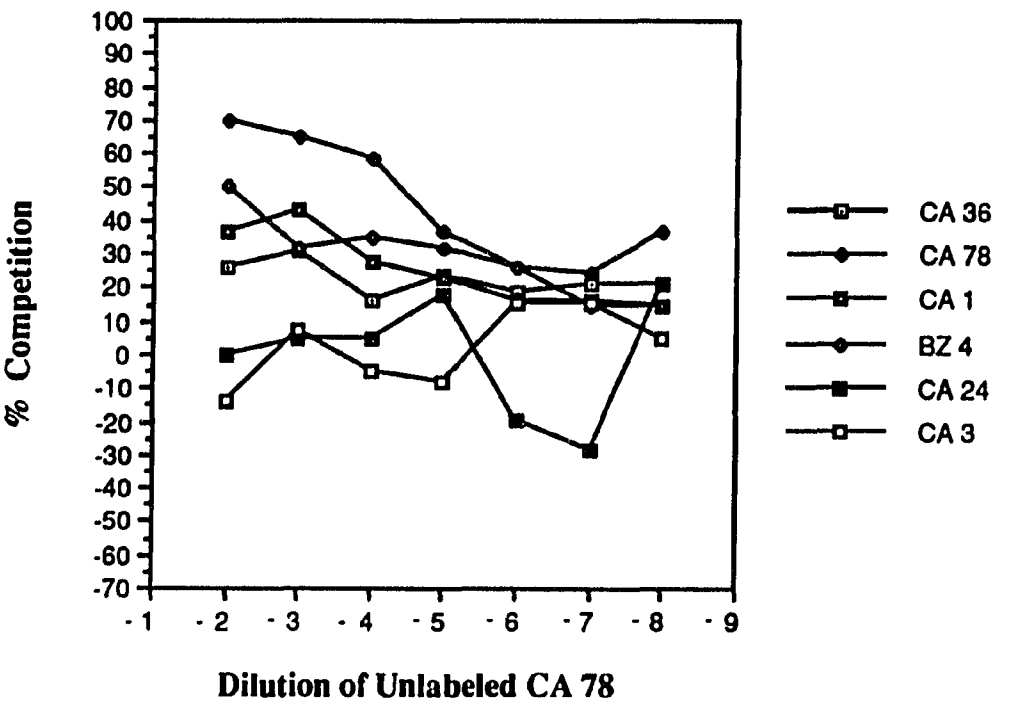
-9
Dilution of Unlabeled CA 1
N2
CA 72
CA 34
CA 78
BZ 4
CA 1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-7
-8
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Dilution of Unlabeled CA 78
CA 36
CA 78
CA 1
BZ 4
CA 24
CA 3

Table 3: Simultaneous Competitive Binding Assays for BVDV-Singer

	Unlabeled Competing Monoclonal Antibody												
^{125}I Mab	Ca 1	Ca 3	N2	Ca82	Ca36	Ca34	Ca39	Ca72	Ca80	BZ-2	BZ-4	Ca78	Ca24
Ca 1	+	+	+	+	+	+	+/-	+	+/-	-	-	-	-
Ca 3	+	+	+	+	+	+	-	+	-	-	-	-	-
N2	+	+	+	+	+	+	+nr	-	-	+	+	-	-
Ca82	+	+	+	+	+	+	+	+	-	+	+	-	-
Ca36	+	+	+	+	+	+	-	+	+	-	-	-	-
Ca34	+	+/-	+	+	+	+	-	-	+/-	+	+	-	-
Ca39	+	+nr	-	+	-	-	+	+	-	-	-	-	-
Ca72	+	+	+nr	+	+	+nr	+	+	-	+	+	-	-
Ca80	+	+nr	+nr	+nr	+	+	+nr	+/-nr	+	-	-	-	-
BZ-2	-	-	+	+	+nr	+	+nr	+/-	-	+	+	-	-
BZ-4	-	-	+	+	+nr	+	+/-	+/-	-	+	+	-	-
Ca78	-	-	-	-	-	-	-	-	-	-	-	+	-
Ca24	-	-	-	-	-	-	-	-	-	-	-	-	-

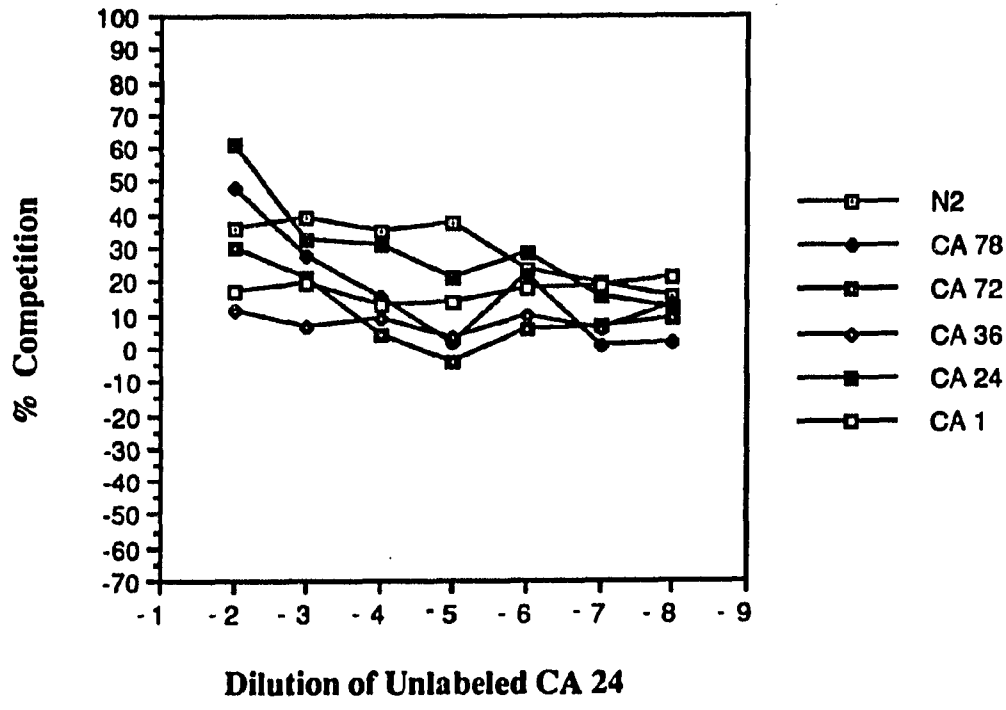
+ = > 60% competition

+/- = 40-60% competition

- = < 30% competition

nr = non-reciprocal competition

Figure 3: Simultaneous competitive binding assays of BVBV-7443 using Ca 24 and Ca 80. Dilutions of labeled purified Mab (50% binding concentration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.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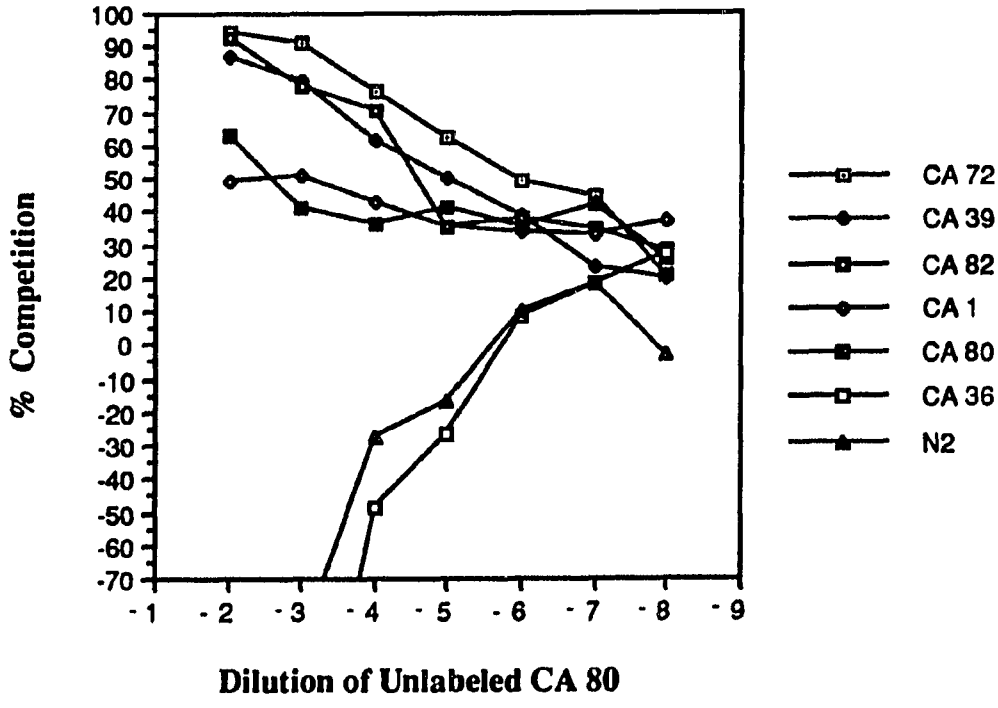
-8
-9
Dilution of Unlabeled CA 24
N2
CA 78
CA 72
CA 36
CA 24
CA 1


% Competition
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-70
-1
-2
-3
-4
-5
-6
-7
-8
-9
Dilution of Unlabeled CA 80
CA 72
CA 39
CA 82
CA 1
CA 80
CA 36
N2

Table 4: Simultaneous Competitive Binding Assay for BVDV-7443

	Unlabeled Competing Monoclonal Antibody												
^{125}I Mab	Ca 1	Ca 3	N2	Ca34	Ca36	Ca72	Ca82	Ca80	Ca39	Ca24	Ca78	BZ-2	BZ-4
Ca 1	+	+	+	+	+	+	+	+/-	+/-	-	-	-	-
Ca 3	+	+	+	+/-	+	+	+	+/-	+/-	-	-	-	-
N2	+	+	+	+	+	+	+	Enr	-	-	-	-	-
Ca34	+	+	+	+	+	+	+	-	+nr	-	-	-	-
Ca36	+	+	+	+	+	+	+	Enr	+/-nr	-	-	-	-
Ca72	+	+	+	+	+	+	+	+	+	-	-	-	-
Ca82	+	+	+	+	+	+	+	+	+	-	-	-	-
Ca80	+	+	+nr	+nr	+nr	+	+	+	+	-	-	-	-
Ca39	+/-	+/-	Enr	Enr	Enr	+	+	+	+	-	-	-	-
Ca24	-	-	-	-	-	-	-	-	-	+	-	-	-
Ca78	-	-	-	-	-	-	-	-	-	-	-	-	-
BZ-2	-	-	-	-	-	-	-	-	-	-	-	-	-
BZ-4	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = > 60% competition

+/- = 40-60% competition

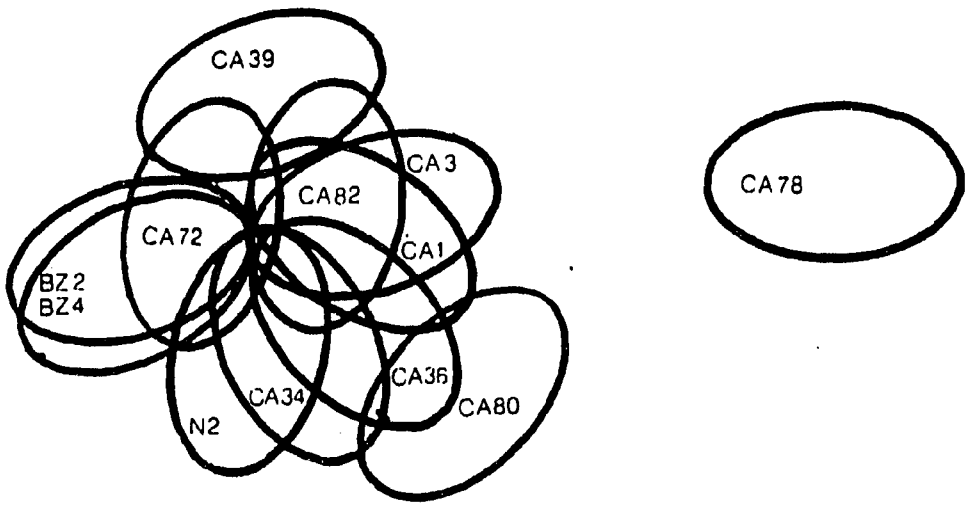
- = < 30% competition

nr = non-reciprocal competition

E = enhancement of binding > 50%

Figure 4: Proposed topographic maps of neutralizing epitopes on gp53 of the Singer cytopathic and 7443 noncytopathic isolates of bovine viral diarrhea virus.

BVDV-Singer



BVDV-7443

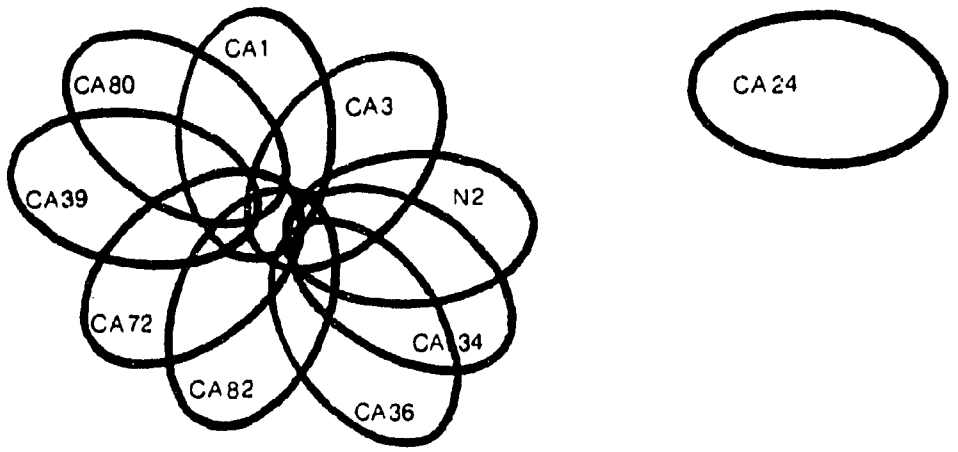


Table 5. Neutralization Titers of Monoclonal Antibodies specific for BVDV in the Presence and Absence of Serum Complement

Monoclonal antibody	Singer		7443	
	C'[1]	No C'	C'	No C'
CA 1	1024[2]	1024	512	512
CA 3	1024	1024	512	512
CA 24	ND[3]	ND	64	32
CA 34	512	256	256	256
CA 36	32	16	256	256
CA 39	1024	1024	512	512
CA 72	<2	<2	512	512
CA 78	64	32	ND	ND
CA 80	1024	1024	256	256
CA 82	1024	1024	512	256
N2	1024	1024	1024	1024
BZ 2	512	512	ND	ND
BZ 4	512	512	ND	ND

[1] Serum complement
[2] Reciprocal of last dilution showing neutralization
[3] Not determined

DISCUSSION

In this study, a panel of thirteen Mabs raised against gp53 of two cytopathic and four noncytopathic isolates of BVDV, that had viral neutralizing activity, were used in serological assays to characterize epitopes on the glycoprotein. Competitive binding assays between pairs of Mabs were performed to elucidate the topographical orientation. Antibody binding assays were used to estimate the relative avidity, and the affinity binding constant of each Mab was determined by regression analysis of the data. In addition, the position of these epitopes relative to carbohydrate moieties, was examined.

Competitive binding assay results are based upon 1-step (simultaneous) assays (figures 2 and 3) which did not differ from 2-step (consecutive) assays. Non-reciprocal competition between antibody pairs was observed (table 3 and 4). Non-reciprocal competition is likely attributable to steric hinderance rather than specific binding of both Mabs to the epitope, therefore, these results were not used in construction of the topographical epitope maps. Potential topographic maps for the neutralizing epitopes on gp53 of the Singer cytopathic and 7443 noncytopathic isolates of BVDV are shown in figure 4. Most of the Mabs bound to a cluster of overlapping epitopes in a single antigenic domain (Domain A) on gp53 of both cytopathic and noncytopathic BVDV virus isolates. A possible second antigenic domain (Domain B) was identified on both cytopathic and noncytopathic BVDV isolates by binding of Mabs Ca 24 and Ca 78. Domain A on the Singer isolate was identified by the binding of eleven Mabs to overlapping epitopes, while eight Mabs bound to overlapping epitopes in domain A of the 7443 isolate. The difference in epitopic models for the cytopathic and noncytopathic BVDV isolates may be due in part to variability in epitopes on the gp53. Kreeft et al. (1990) have described rapid antigenic change in a BVDV vaccine virus and hypothesize the possibility of a virus mechanism that may cause epitopes to

disappear or emerge. There is a possibility that emergence of undetected epitopes may have altered the topography of the detected overlapping epitopes in domain A of both isolates.

The influence of serum complement on neutralization of BVDV by this panel of Mabs, and further potential delineation of the epitopes recognized by the Mabs, was also examined by determining the neutralization titers of each Mab in the presence and absence of serum complement (table 5). The activation of the full complement cascade can effect irreversible damage to the envelope of a virus (Oldstone, 1975; Sissons and Oldstone, 1980). However, complement-effected lysis does not necessarily follow from complement activation. If antibody reacts with an epitope near the extremity of envelope proteins, the activated components of complement may lose their ability to bind before diffusing to the viral envelope. Secondly, complement-mediated damage may occur but fail to produce lysis if the density of epitopes on the virus is too low. The neutralization titer of each Mab in this panel was neither enhanced nor reduced, indicating that these epitopes were likely located near the free end of the viral glycoprotein.

Endoglycosidase treatment of BVDV glycoproteins has been used to remove carbohydrate moieties from those proteins (Collett et al., 1988b). The topographical orientation of epitopes on the 53 kd glycoprotein was further analyzed by binding assays following treatment of viral glycoprotein with endoglycosidase H and F/N-glycosidase F. The binding of each antibody was either greatly reduced or prevented by the removal of carbohydrate from the glycoprotein (data not shown). These results might indicate that the neutralizing epitopes of BVDV are located in the portion of the glycoprotein that carries the carbohydrate moites. However, similar results with other viruses have lead to the hypothesis that removal of carbohydrate chains alters the three-dimensional structure of a protein, and may influence a distant antigenic site (Bruck et al., 1982). A procedure not done in this study that might provide additional information on the location of epitopes would be

propagation of BVDV in the presence of the n-linked glycosylation inhibibitor, tunicamycin, and testing for binding by the Mab panel.

Mateo Rosell (1988) and Moennig et al. (1989) have previously describe topographic analysis of BVDV gp53 epitopes, and determined the existence of at least four antigenic domains containing overlapping epitopes, using an immunoperoxidas assay. Our results of CBA's using radiolabeled Mabs extend the previous findings, and further reinforce the model of a limited number of neutralizing antigenic domains with overlapping epitopes. Characterization and topographical analysis of hog cholera virus epitopes (Wensvoort, 1989; Wensvoort et al., 1990; Greiser-Wilke et al., 1990) have also indicated the presence of at least three overlapping neutralizing antigenic domains.

Antigenic analyses of proteins with Mabs is an important and useful tool. However, a finite panel of antibodies may only provide information about a limited number of epitopes. Proteins may carry a large number of antigenically reactive sites (Benjamin et al, 1984). However, Arnon (1980) has suggested that only a limited number of potential antigenic sites are immunodominant and can stimulate a host's immune response. In this study, domain A appears to be an immuno-dominant antigenic domain on gp53, but this may be a reflection of the panel of Mabs used. Mabs against BVDV are difficult to produce. This may be attributable to the difficulty in viral purification and/or immunogenicity of the viral proteins. The relatively limited number of Mabs available for this study may have influenced the results. The true, general antigenic structure of gp53 may only be assessed with large, diverse panels of Mabs .

In conclusion, the topographical orientation of neutralizing epitopes on the 53 kd envelope glycoprotein of BVDV was determined. At least two distinct antigenic domains were identified, made up of epitopes that are conformationally-dependent in nature. Virus neutralization at these epitopes is not complement-dependent. Furthermore, endoglycosidase

treatment indicates the epitopes appear to be located in or near the carbohydrate moieties of the glycoprotein. A high degree of antigenic mutation in BVDV has been observed. Results presented in this study are in accordance with those observations.

REFERENCES

Arnon, R. 1980. Chemically defined antiviral vaccines. Annu. Rev. Microbiol. 34:593-618.

Benjamin, D. C., J. A. Berzofsky, I. J. East, F. R. N. Gurd, C. Hannun, S. J. Leach, E. Margoliash, J. G. Michael, A. Miller, E. M. Prager, M. Reichlin, E. E. Sercarz, S. J. Smith-Gill, P. E. Todd, and A. C. Wilson. 1984. The antigenic structure of proteins: A reappraisal. Annu. Rev. Immunol. 2:67-101.

Bolin, S. , V. Moennig, N. E. Kelso Gourley, and J. Ridpath. 1988. Monoclonal antibodies with neutralizing activity segregate isolates of bovine viral diarrhea virus into groups. Arch. Virol. 99:117-123.

Bruck, C., D. P ortetelle, A. Burny, and J. Zavada. 1982. Topological analysis by monoclonal antibodies of BLV-gp51 epitopes involved in viral functions. Virology. 122:342-352.

Collett, M. S., R. Larson, C. Gold, D. Strick, D. K. Anderson, and A. F. Purchio, A. F. 1988a. Molecular cloning and nucleotide sequence of pestivirus bovine viral diarrhea virus. Virology 165:191-199.

Collett, M. S., R. Larson, S. K. Belzer, and E. Retzel. 1988b. Proteins encoded by bovine viral diarrhea virus: The genomic organization of a pestivirus. Virology 165:200-208.

Corapi, W. V., R. O. Donis, and E. J. Dubovi. 1988. Monoclonal antibody analyses of cytopathic and noncytopathic viruses from fatal bovine viral diarrhea virus infections. J. Virol. 62(8):2823- 2827.

Darbyshire, J. H. 1960. A serological relationship between swine fever and mucosal disease of cattle. Vet. Rec. 72:331.

Darbyshire, J. H. 1962. Agar gel diffusion studies with a mucosal disease of cattle. III. A serological relationship between a mucosal disease and swine fever virus. Res. Vet. Sci. 3(2):125-128.

Donis, R. O., and E. J. Dubovi. 1987. Glycoproteins of bovine viral diarrhoea virus-mucosal disease virus in infected bovine cells. J. Gen. Virol. 68:1607-1616.

Donis, R. O., W. Corapi, and E. J. Dubovi. 1988. Neutralizing monoclonal antibodies to bovine viral diarrhoea virus bind to the 56K to 58K protein. J. Gen Virol. 69:77-86.

Fernelius, A. L. W. C. Amtower, and W. A. Malmquist. 1973. Bovine viral diarrhea virus in swine: neutralizing antibody in naturally and experimentally infected swine. Can. J. Comp. Med. 37:96-102.

Fraker, P. J. and J. C. Speck. 1978. Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. Biochem. Biophys. Res. Commun. 80:849-857.

Frankel, M. E., and W. Gerhard. 1979. The rapid determination of binding constants for antiviral antibodies by a radioimmunoassay. An analysis of the interaction between hybridoma proteins and influenza virus. Mol. Immunol. 16:101-106.

Greiser-Wilke, I., V. Moennig, C. O. Z. Coulibaly, J. Dahle, L. Leder, and B. Liess. 1990. Identification of conserved epitopes on a hog cholera virus protein. Arch. Virol. 111:213-225.

Horzinek, M. C. 1981. Nonarbo togavirus infections of animals: comparative aspects and diagnosis. Pp.442-478. In Comparative Diagnosis of Viral Diseases. Edited by E. Kurstak and C. Kurstak. New York: Academic Press.

Kreeft, H. A. J. G., I. Greiser-Wilke, V. Moennig, and M. C. Horzinek. 1990. Attempts to characterize bovine viral diarrhea virus isolated from cattle after immunization with a contaminated vaccine. Dstch. tierazl. Wschr. 97:57-104.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-686.

Liess, B., H. R. Frey, and D. Prager. 1977. Antibody response of pigs following experimental infections with strains of hog cholera, bovine viral diarrhea. In: Hog Cholera - Classical Swine Fever and African Swine Fever. Com. Euro. Commun. Eur 5904, pp. 200-211.

Magar, R., H. C. Minocha, and J. Lecomte. 1988. Bovine viral diarrhea virus proteins: Heterogeneity of cytopathogenic and non-cytopathogenic strains and evidence of a 53k Glycoprotein neutralization epitope. Vet. Microbiol. 16:303-314.

Mateo Rosell, A. M. 1988. A Topological and functional map of epitopes on the major surface glycoprotein of the bovine viral diarrhoea virus (BVDV). Vet. Med.Diss. Hanover (thesis).

Matthews, R. E. F. 1982. Classification and nomenclature of viruses. Mathews, ed. 4th Report of the International Committee on Taxonomy of Viruses. Karger Basel.

Moennig, V., A. Mateo, I. Greiser-Wilke, S. R. Bolin, N. E. Kelso, and B. Liess. 1989. A topological and functional epitope map of the surface of bovine viral diarrhea virus. Abstr. Ann. Meet. Amer. Soc. for Microbiol. p. 390. New Orleans, LA, 14-18 May 1989.

Oldstone, M. B. 1975. Virus neutraliztion and virus-induced immune complex disease. Virus-antibody union resulting in immunoprotection or immunologic injury - two sides of the same coin. Prog. Med. Virol. 19:84-119.

Osburn, B. I., G. L. Clarke, and W. C. Stewart. 1973. Border disease-like syndrome in lambs: antibodies to hog cholera and bovine viral diarrhea viruses. J. Am. Vet. Med. Assoc. 163:1165-1167.

Peters, W., I. Greiser-Wilke, V. Moennig, and B. Liess. 1986. Preliminary serological characterization of bovine viral diarrhea virus strains using monoclonal antibodies. Vet. Microbiol. 12:195-200.

Pocock, D. H., C. J. Howard, M. C. Clarke, and J. Brownlie. 1987. Variation on the intracellular polypeptide profiles from different isolates of bovine virus diarrhea virus. Arch. Virol. 94:43-53.

Ridpath, J. F., T. L. Lewis, S. R. Bolin, and E. S. Berry. 1991. Antigenic and genomic comparison between non-cytopathic and cytopathic bovine viral diarrhoea viruses isolated form cattle that had spontaneous mucosal disease. J. Gen. Virol. 72:725-729.

Saravis, C. A. 1984. Improved blocking of nonspecific antibody binding sites on nitrocellulose membranes. Electrophoresis 5:54-55.

Sissons, J. G., and M. B. Oldstone. 1980. Antibody-mediated destruction of virus-infected cells. Adv. Immunol. 29:209-260.

Taggart, R. T., and I. M. Samloff. 1983. Stable antibody-producing murine hybridomas. Science 219:1228-1230.

Wensvoort, G. 1989. Topographical and funcional mapping of epitopes on hog cholera virus with monoclonal antibodies. J. Gen. Virol. 70:2865-2876.

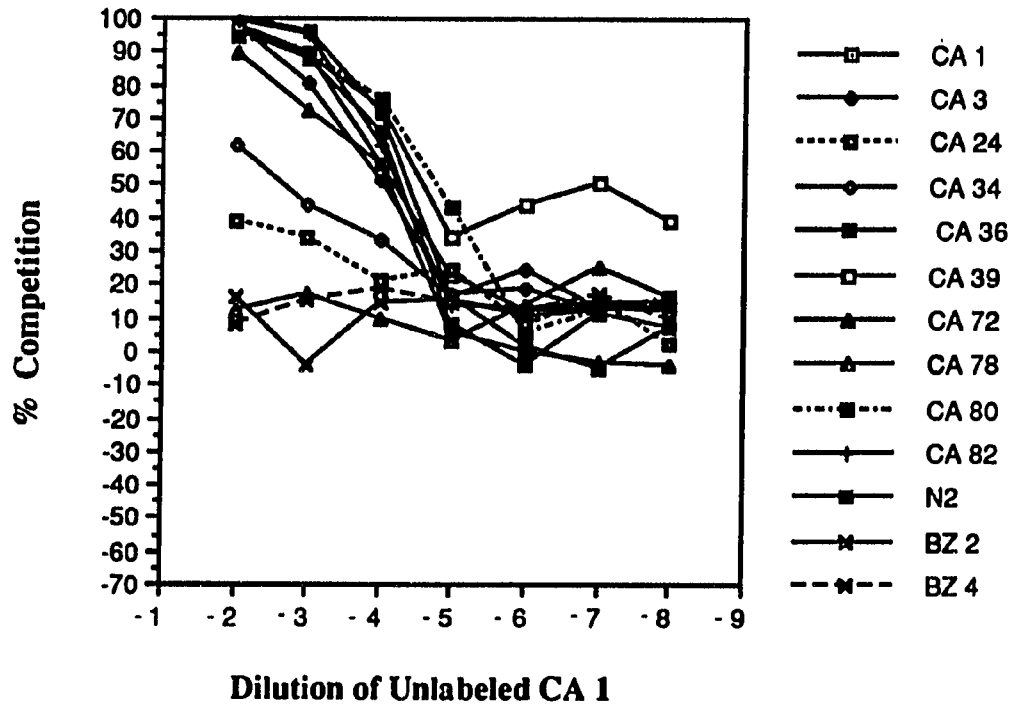
Wensvoort, G., J. Boonstra, and B. G. Bodzingz. 1990. Immunoaffinity purification and characterization of the envelope protein E1 of hog cholera virus. J. Gen. Virol. 71:531-540.

Westaway, E. G., M. A. Brinton, S. Giadamovich, M. C. Horzinek, A. Igarashi, L. Kataritainen, D. K. Lvov, J. S. Porterfield, and D. W. Trent. 1985. Togaviridae. Intervirology 24:125-139.

Yewdell, J. W. and W. Gerhard. 1981. Antigenic characterization of viruses by monoclonal antibodies. Ann. Rev. Microbiol. 35:185-206.

APPENDIX A: GRAPHS

Figure 5: Simultaneous competitive binding assays of BVBV-Singer using Ca 1 and Ca 3. Dilutions of labeled purified Mab (50% binding concentration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.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80
70
60
50
40
30
20
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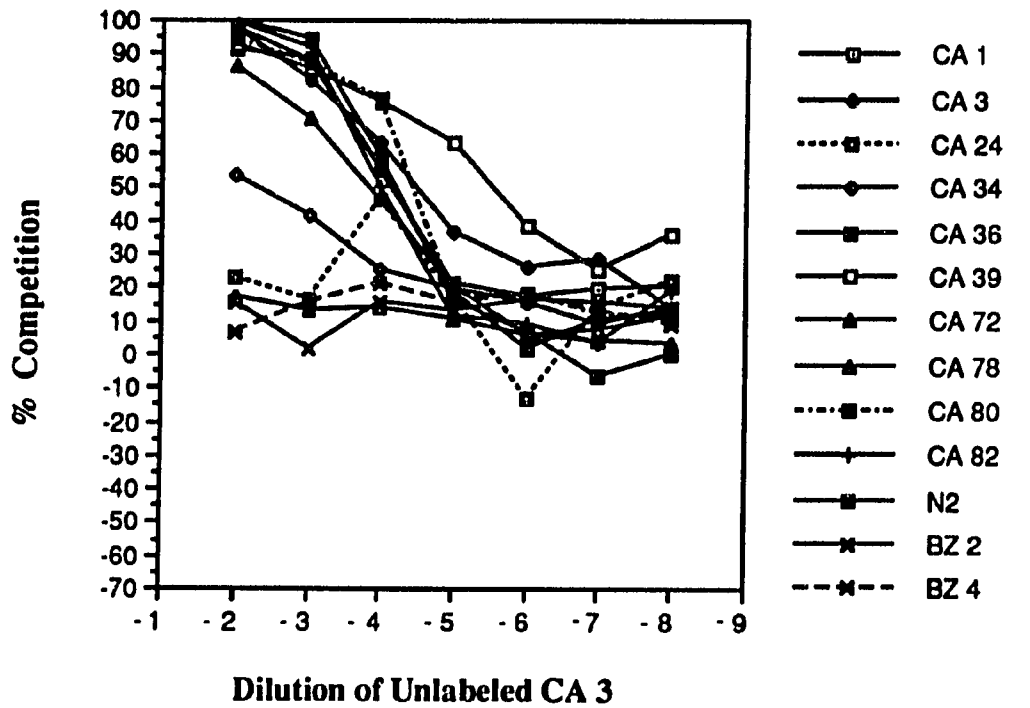
0
-10
-20
-30
-40
-50
-60
-70
-1
-2
-3
-4
-5
-6
-7
-8
-9
Dilution of Unlabeled CA 1
CA 1
CA 3
CA 24
CA 34
CA 36
CA 39
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4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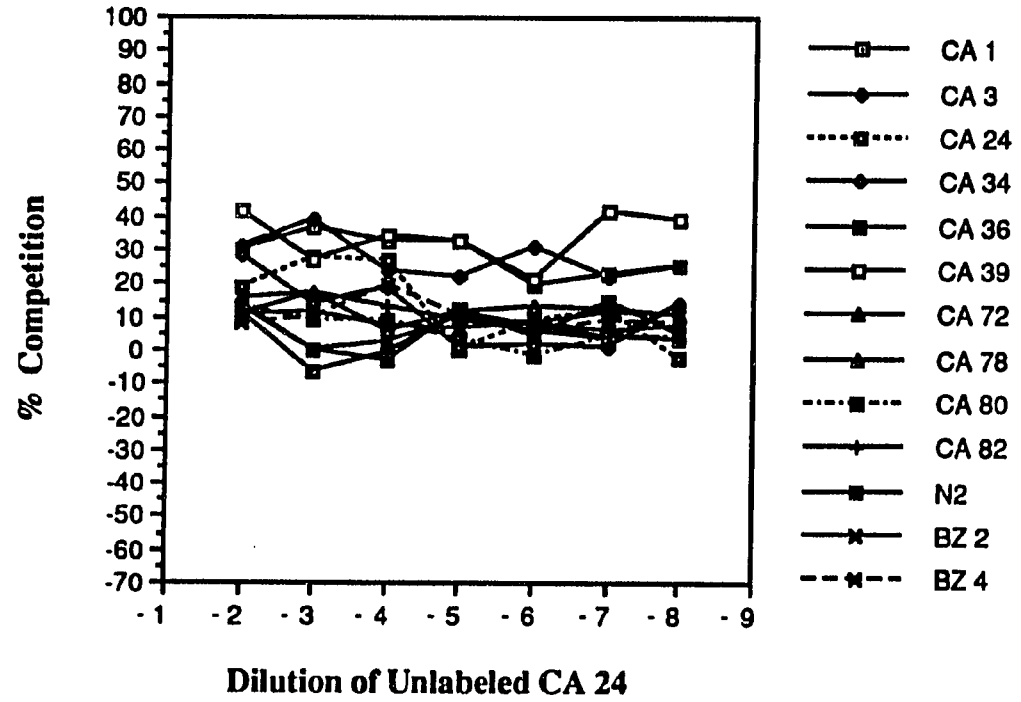
-9
Dilution of Unlabeled CA 3
CA 1
CA 3
CA 24
CA 34
CA 36
CA 39
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4

Figure 6: Simultaneous competitive binding assays of BVBV-Singer using Ca 24 and Ca 34. Dilutions of labeled purified Mab (50% binding concentration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.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-3
-4
-5
-6
-7
-8
-9
Dilution of Unlabeled CA 24
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CA 36
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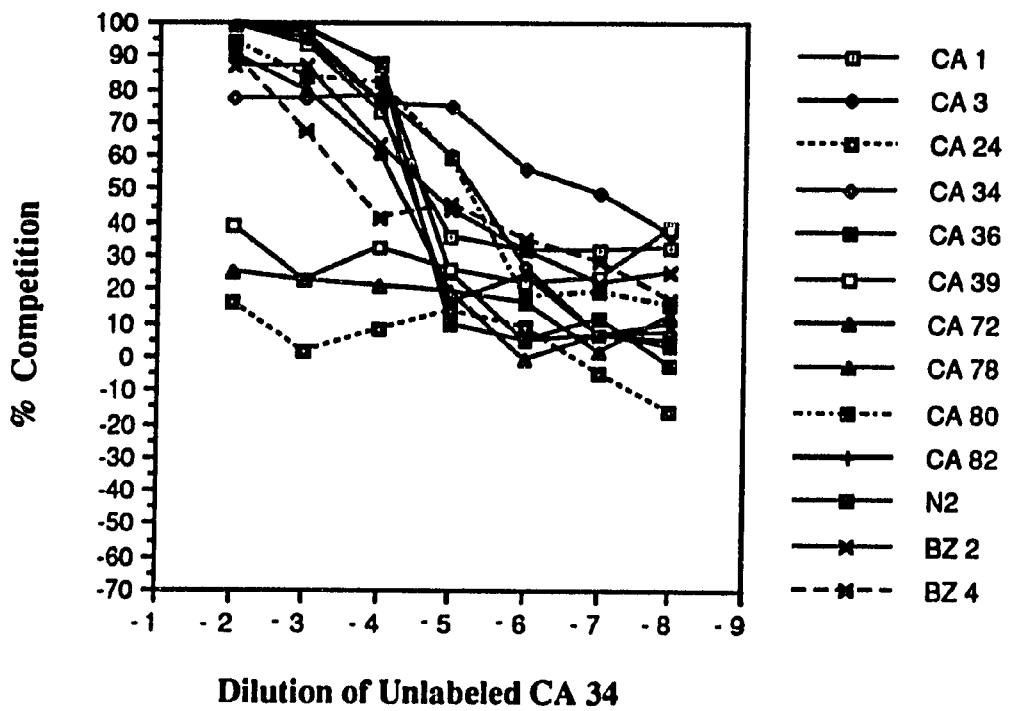
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4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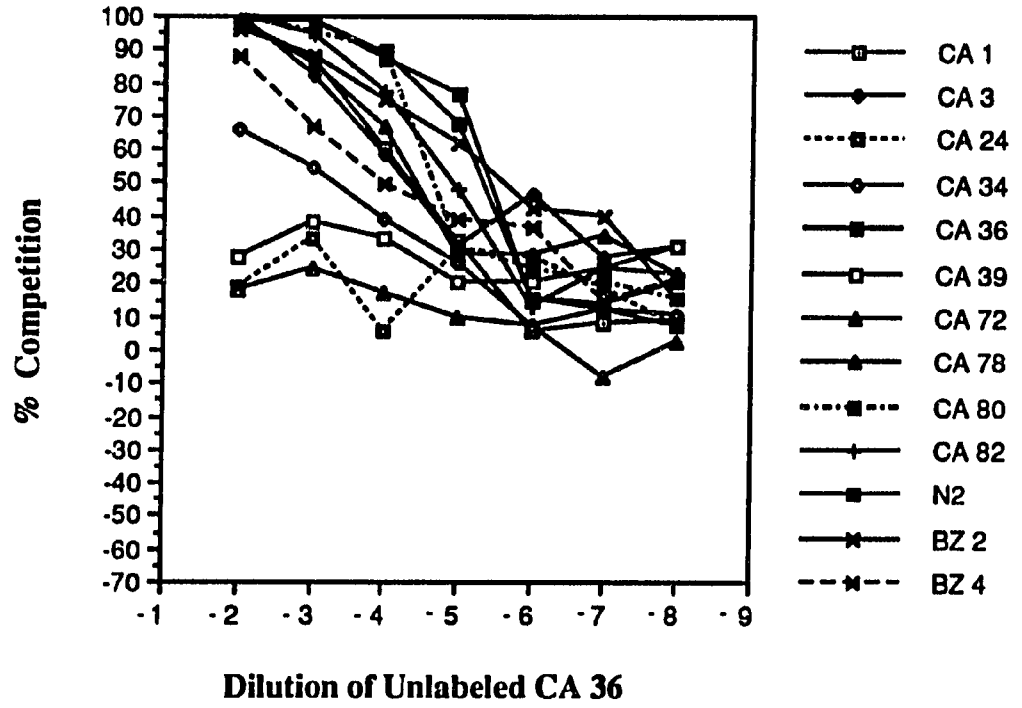
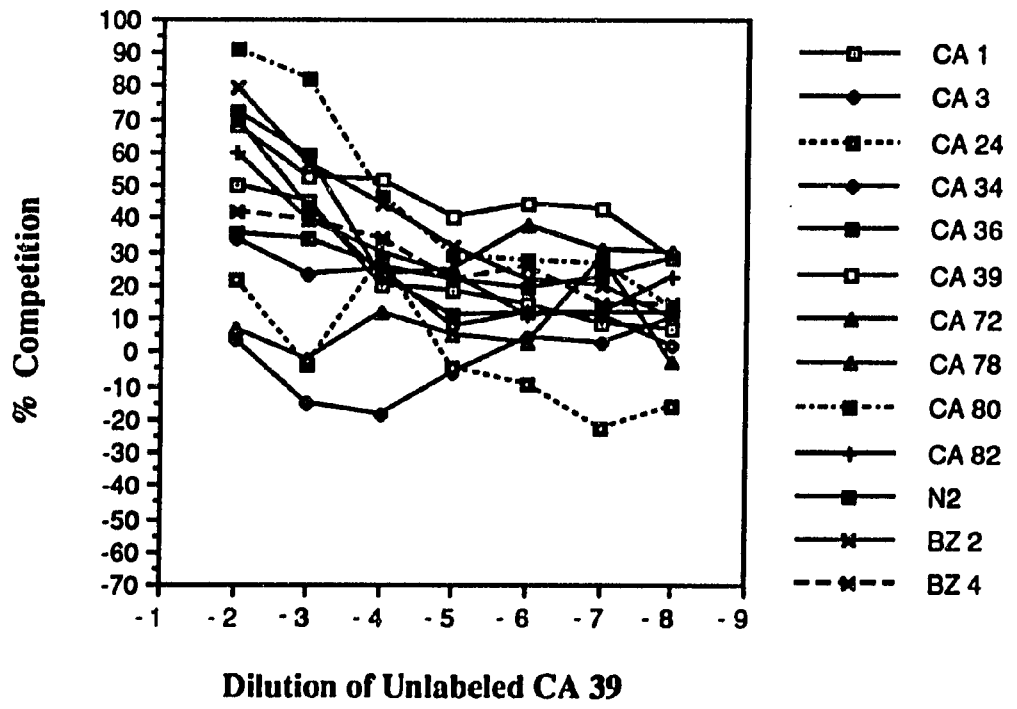
-9
Dilution of Unlabeled CA 34
CA 1
CA 3
CA 24
CA 34
CA 36
CA 39
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4

Figure 7: Simultaneous competitive binding assays of BVBV-Singer using Ca 36 and Ca 39. Dilutions of labeled purified Mab (50% binding concentration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.

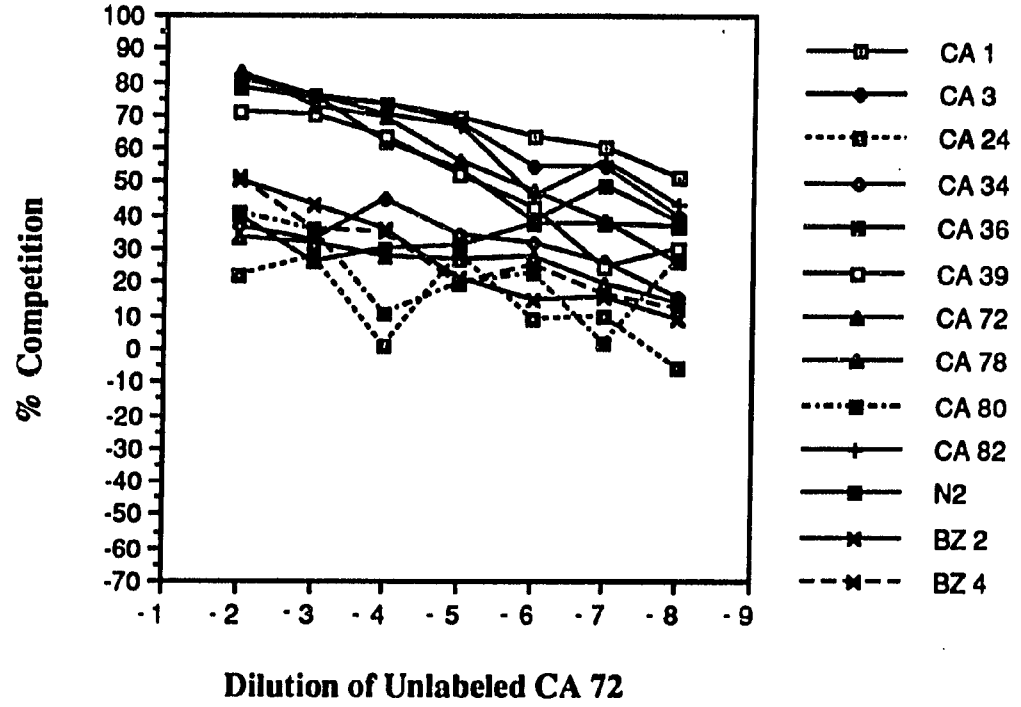


Dilution of Unlabeled CA 36



Dilution of Unlabeled CA 39

Figure 8: Simultaneous competitive binding assays of BVBV-Singer using Ca 72 and Ca 78. Dilutions of labeled purified Mab (50% binding concentration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.



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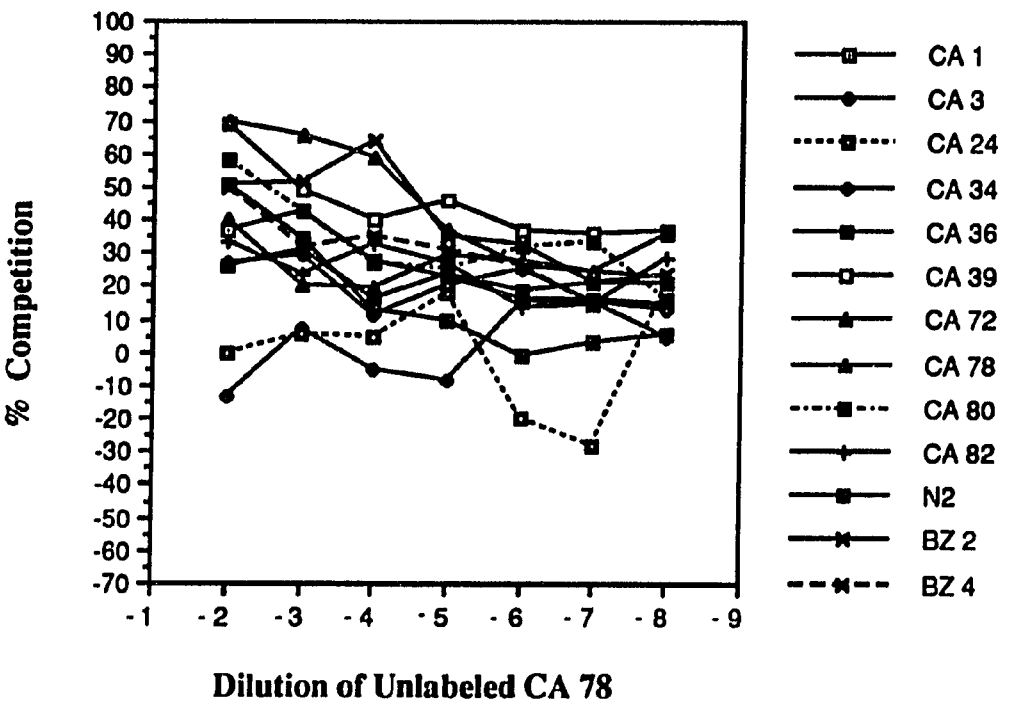
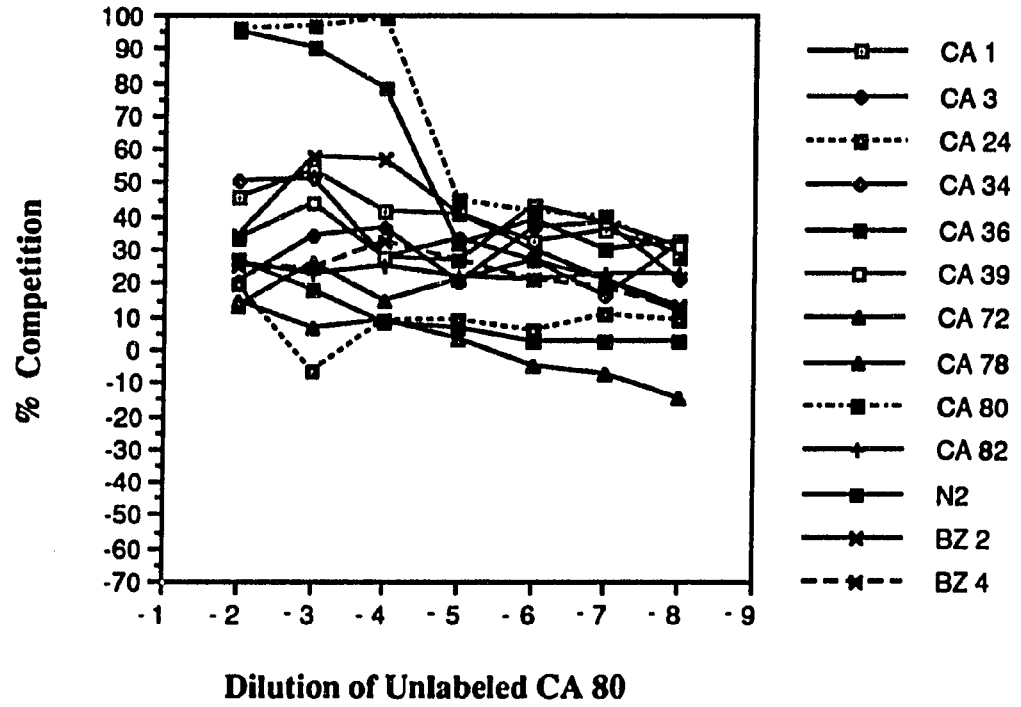


Dilution of Unlabeled CA 78

Figure 9: Simultaneous competitive binding assays of BVBV-Singer using Ca 80 and Ca 82. Dilutions of labeled purified Mab (50% binding concentration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.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Dilution of Unlabeled CA 80
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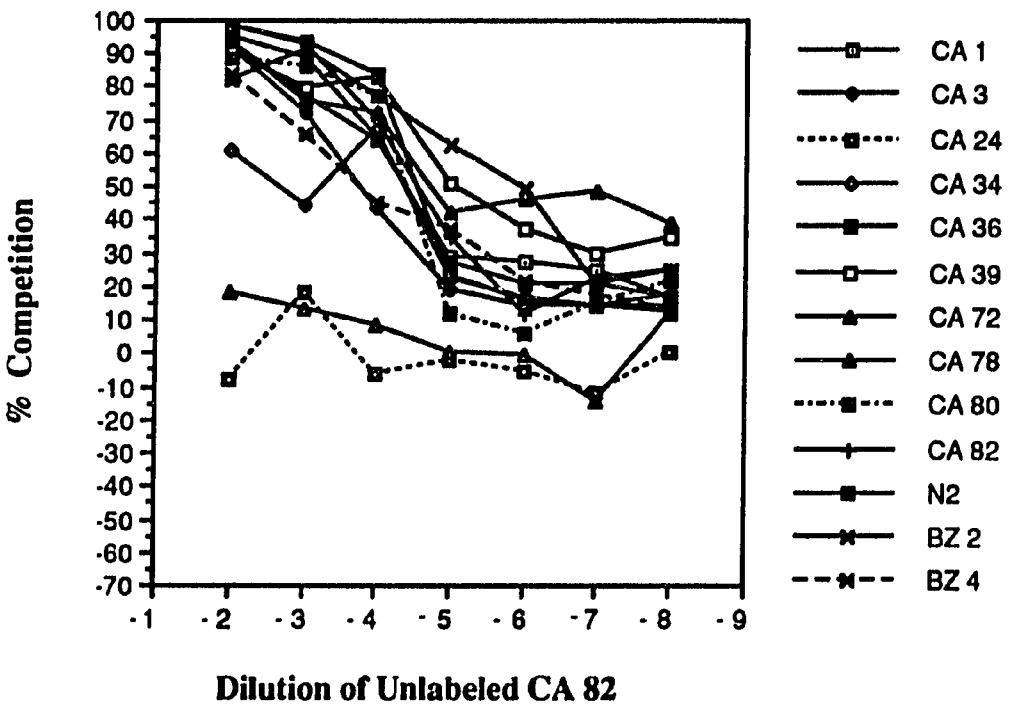
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4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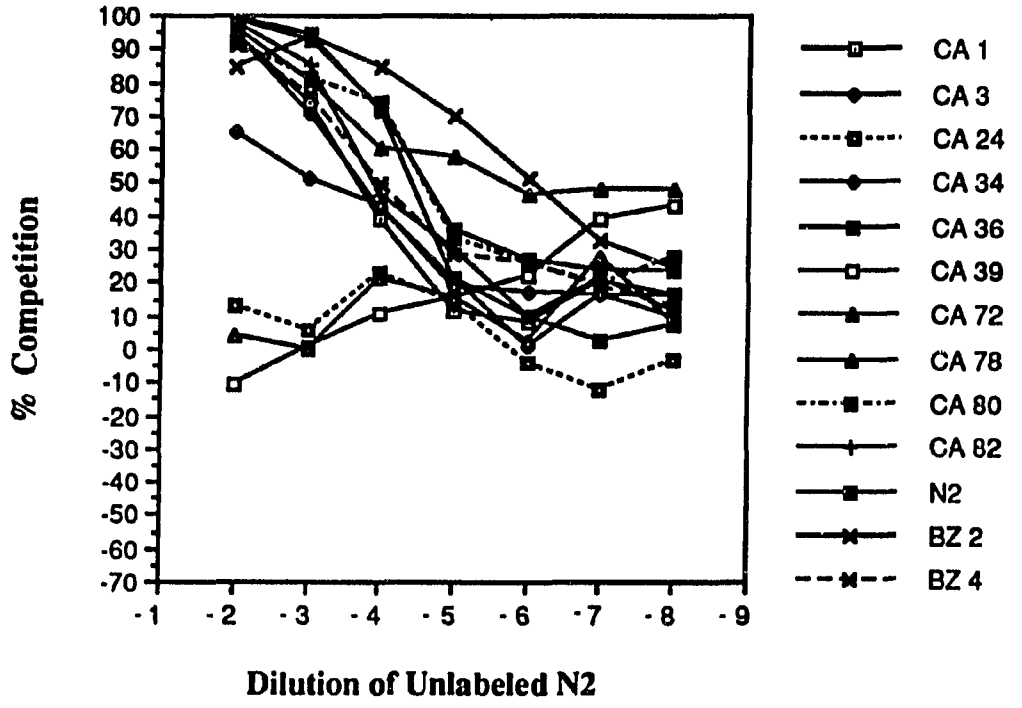
-9
Dilution of Unlabeled CA 82
CA 1
CA 3
CA 24
CA 34
CA 36
CA 39
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4

Figure 10: Simultaneous competitive binding assays of BVBV-Singer using N2 and BZ-2. Dilutions of labeled purified Mab (50% binding concen-ration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.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Dilution of Unlabeled N2
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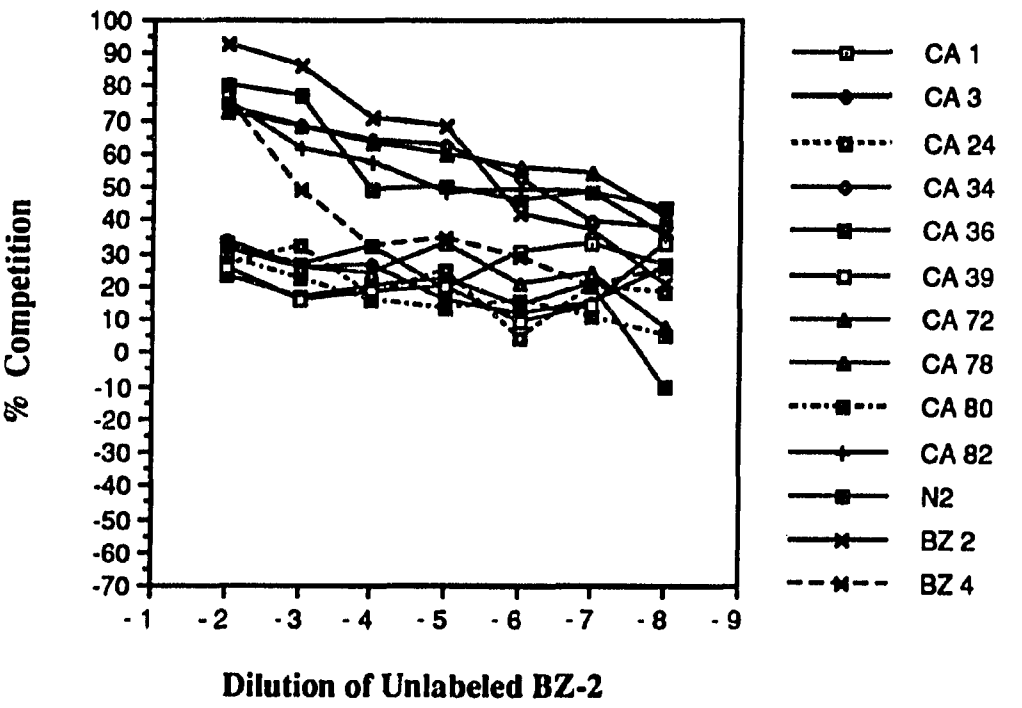
CA 82
N2
BZ 2
BZ 4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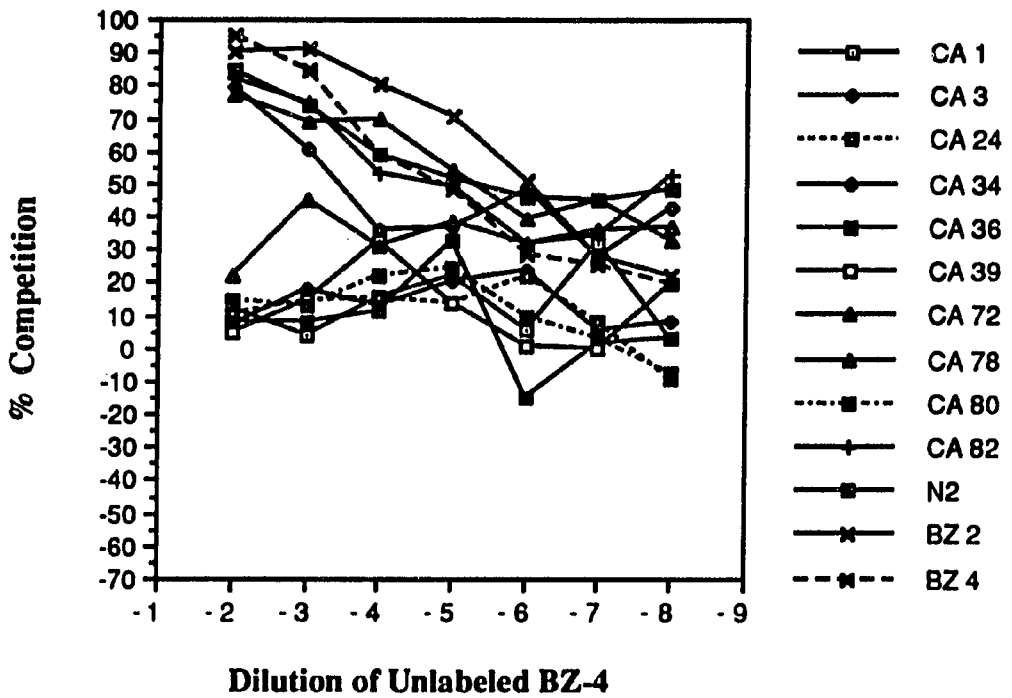
-9
Dilution of Unlabeled BZ-2
CA 1
CA 3
CA 24
CA 34
CA 36
CA 39
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4

Figure 11: Simultaneous competitive binding assays of BVBV-Singer using BZ-4. Dilutions of labeled purified Mab (50% binding concentration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.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-2
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-4
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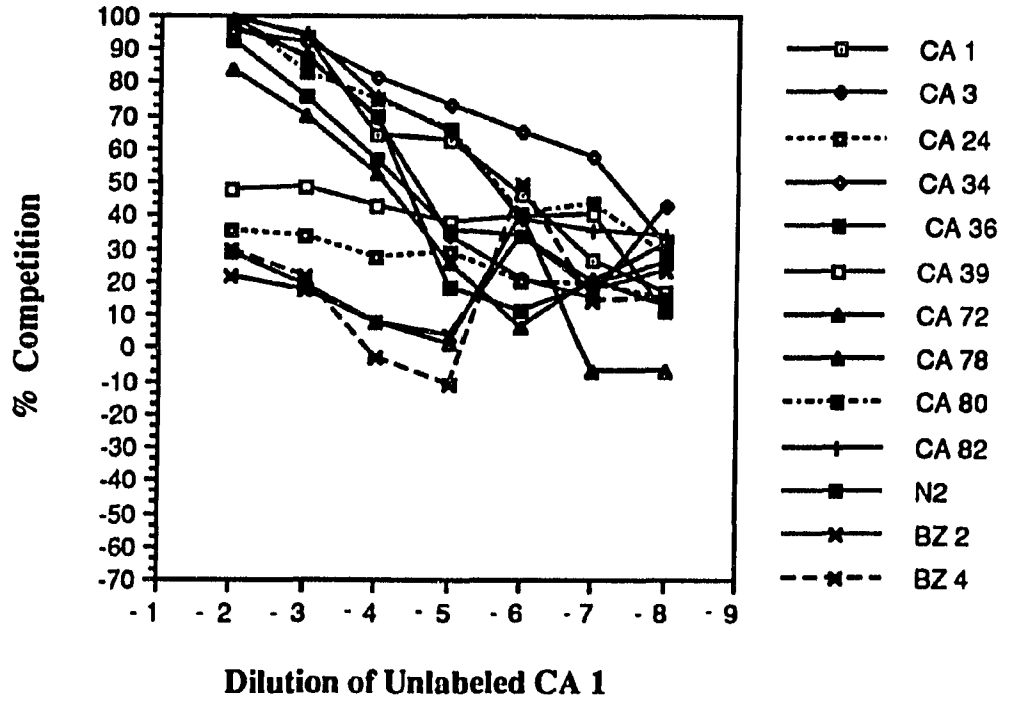
-7
-8
-9
Dilution of Unlabeled BZ-4
CA 1
CA 3
CA 24
CA 34
CA 36
CA 39
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4

Figure 12: Simultaneous competitive binding assays of BVBV-7443 using Ca 1 and Ca 3. Dilutions of labeled purified Mab (50% binding concentration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.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-9
Dilution of Unlabeled CA 1
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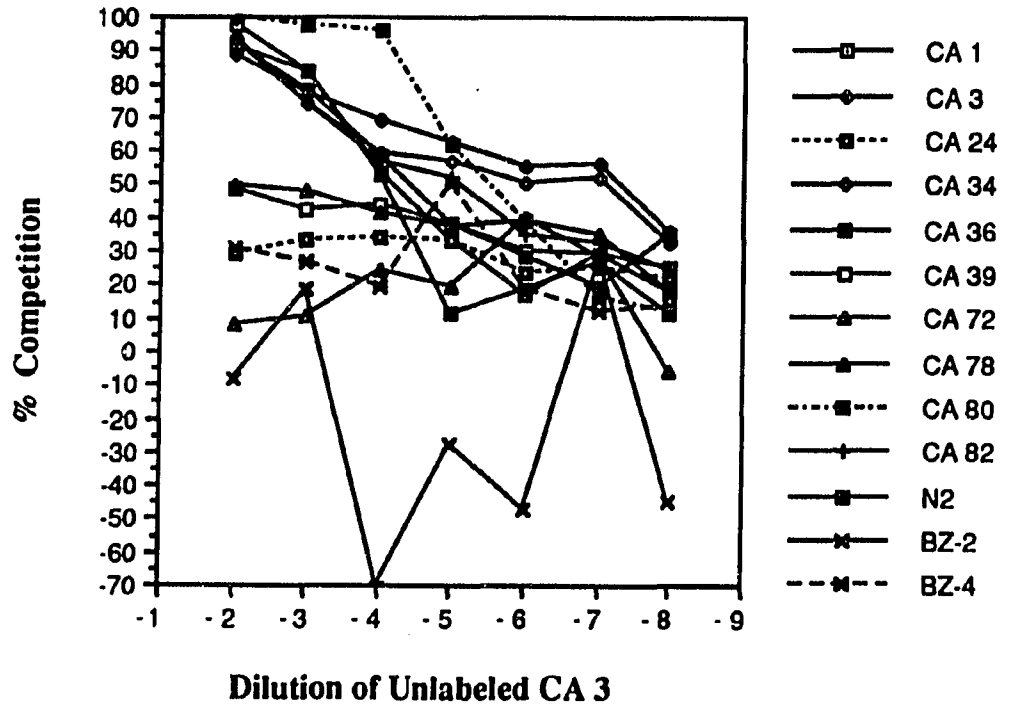
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4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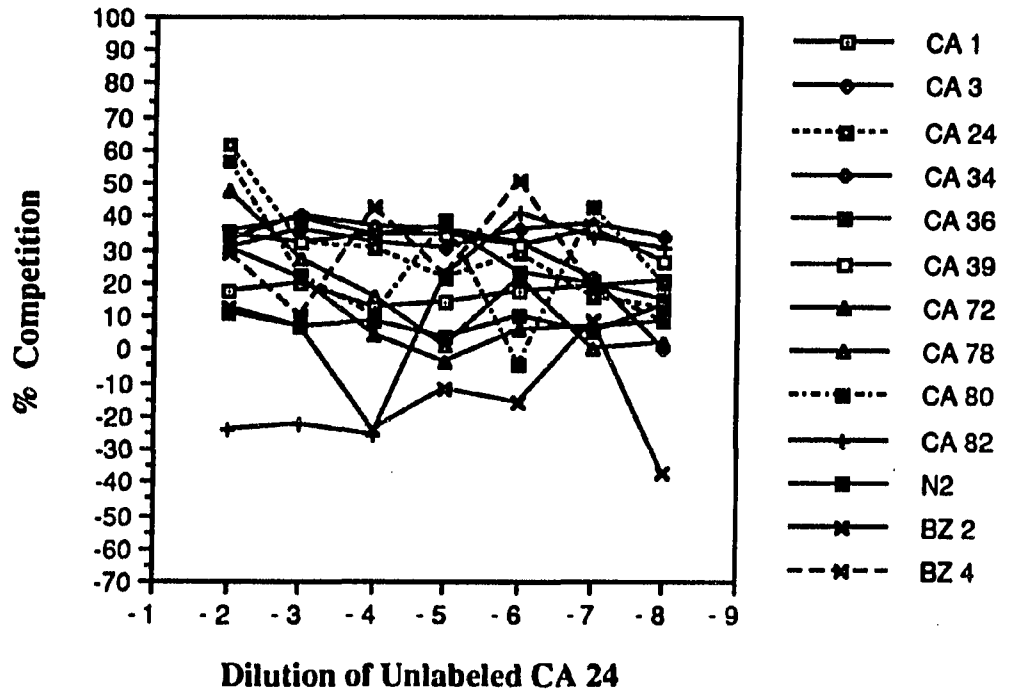
-3
-4
-5
-6
-7
-8
-9
Dilution of Unlabeled CA 3
CA 1
CA 3
CA 24
CA 34
CA 36
CA 39
CA 72
CA 78
CA 80
CA 82
N2
BZ-2
BZ-4

Figure 13: Simultaneous competitive binding assays of BVBV-7443 using Ca 24 and Ca 34. Dilutions of labeled purified Mab (50% binding concentration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.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-1
-2
-3
-4
-5
-6
-7
-8
-9
Dilution of Unlabeled CA 24
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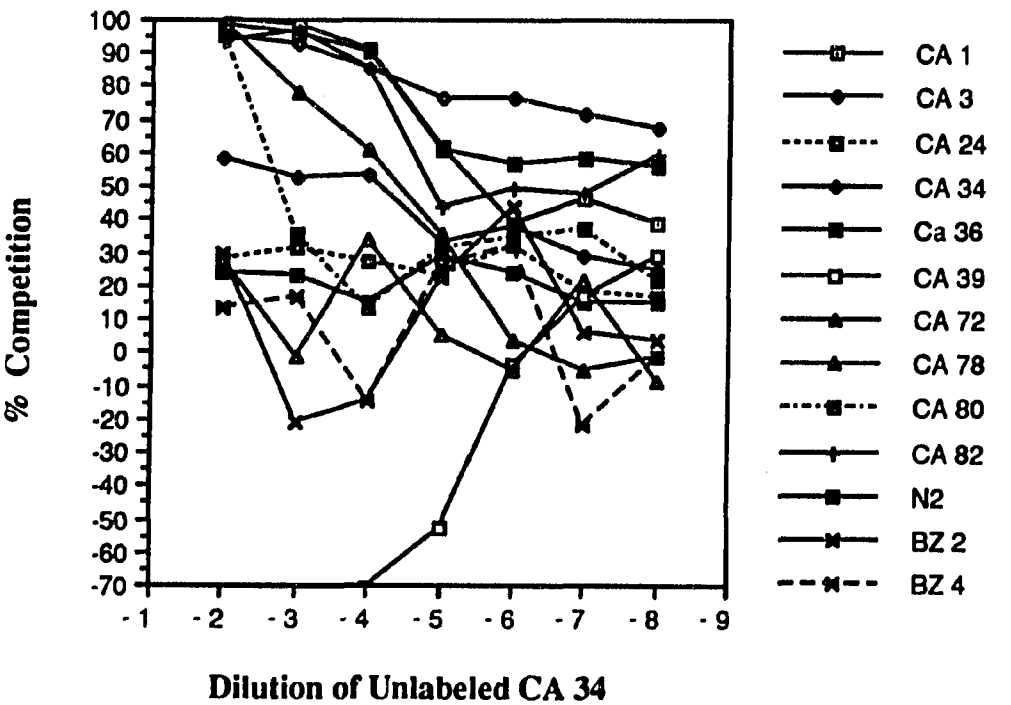
CA 80
CA 82
N2
BZ 2
BZ 4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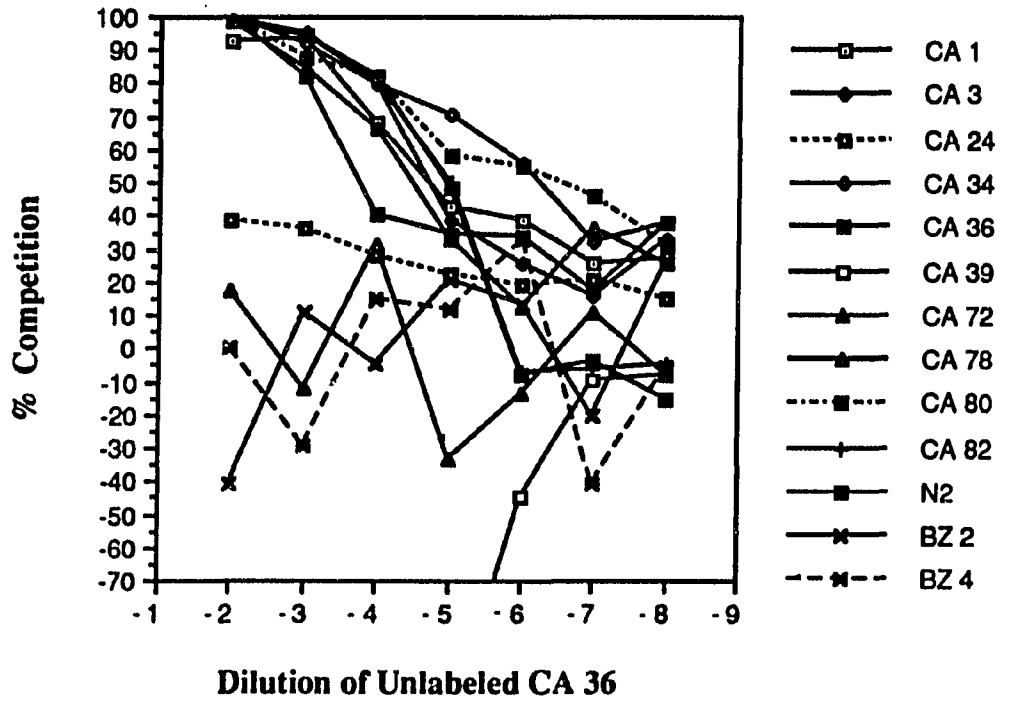
-9
Dilution of Unlabeled CA 34
CA 1
CA 3
CA 24
CA 34
Ca 36
CA 39
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4

Figure 14: Simultaneous competitive binding assays of BVBV-7443 using Ca 36 and Ca 39. Dilutions of labeled purified Mab (50% binding concentration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.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-1
-2
-3
-4
-5
-6
-7
-8
-9
Dilution of Unlabeled CA 36
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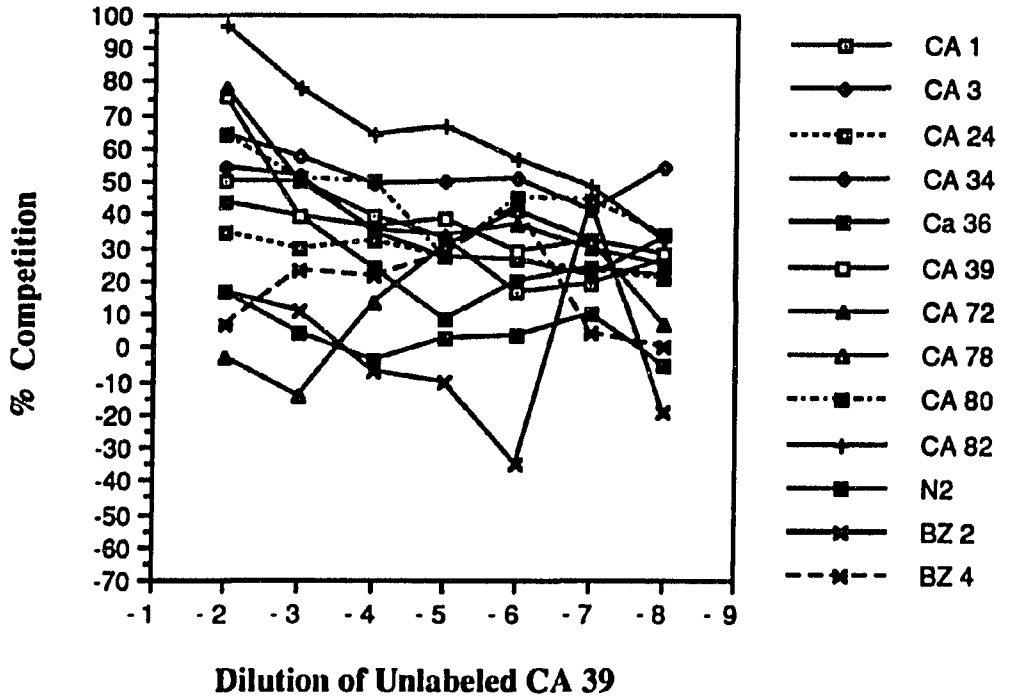
CA 34
CA 36
CA 39
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4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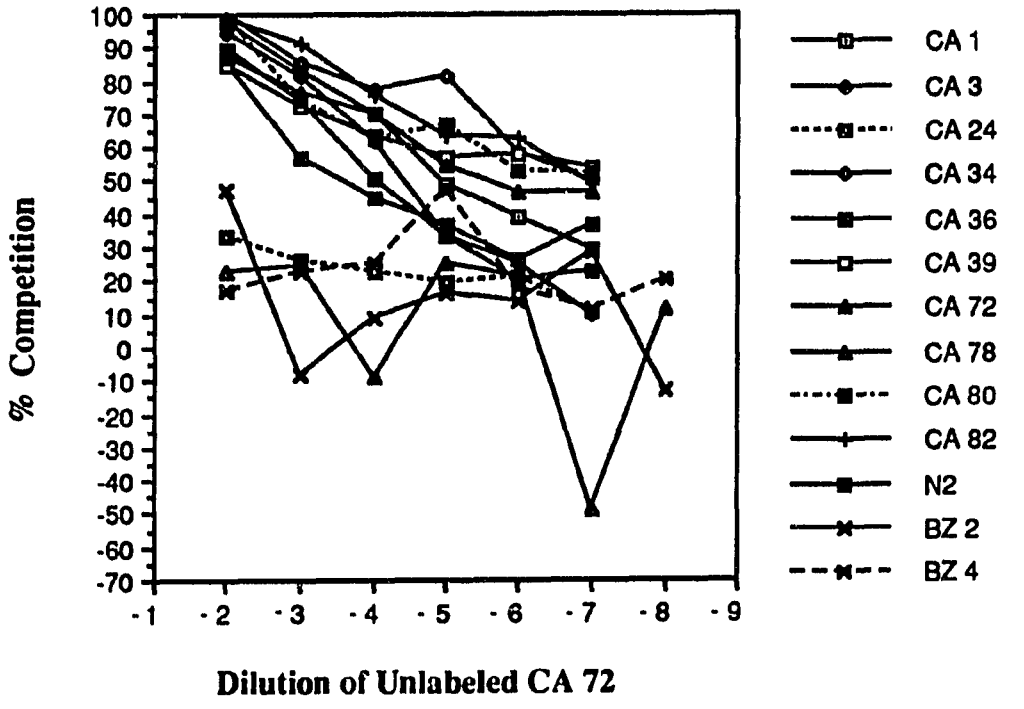
-9
Dilution of Unlabeled CA 39
CA 1
CA 3
CA 24
CA 34
Ca 36
CA 39
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4

Figure 15: Simultaneous competitive binding assays of BVBV-7443 using Ca 72 and Ca 78. Dilutions of labeled purified Mab (50% binding concentration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.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Dilution of Unlabeled CA 72
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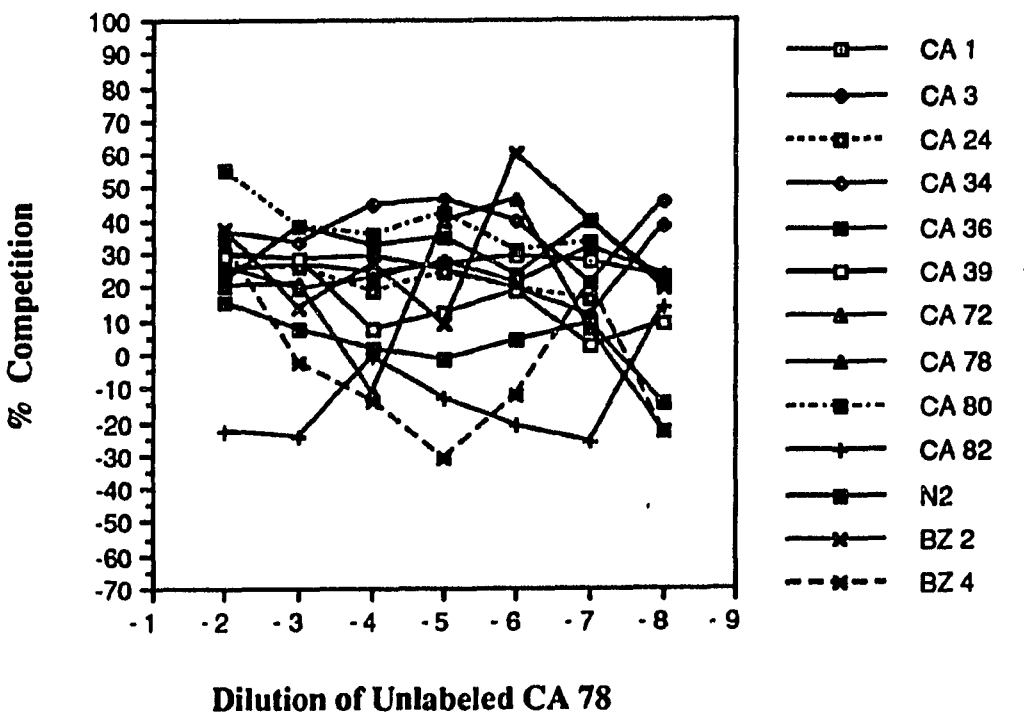
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4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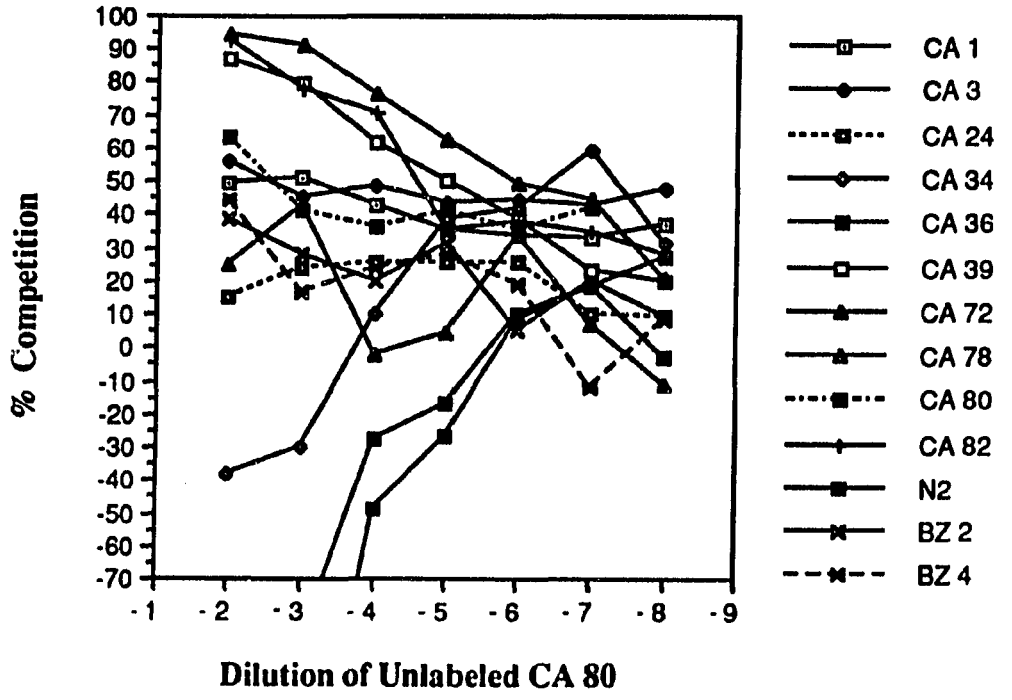
-9
Dilution of Unlabeled CA 78
CA 1
CA 3
CA 24
CA 34
CA 36
CA 39
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4

Figure 16: Simultaneous competitive binding assays of BVBV-7443 using Ca 80 and Ca 82. Dilutions of labeled purified Mab (50% binding concentration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.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-6
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-9
Dilution of Unlabeled CA 80
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CA 72
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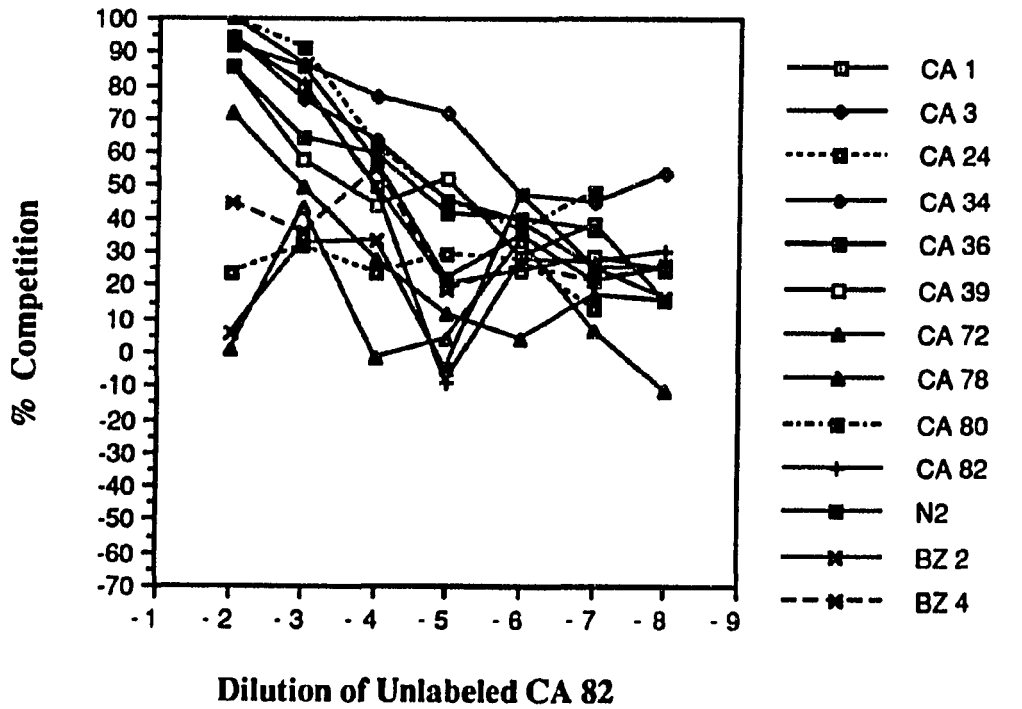
CA 80
CA 82
N2
BZ 2
BZ 4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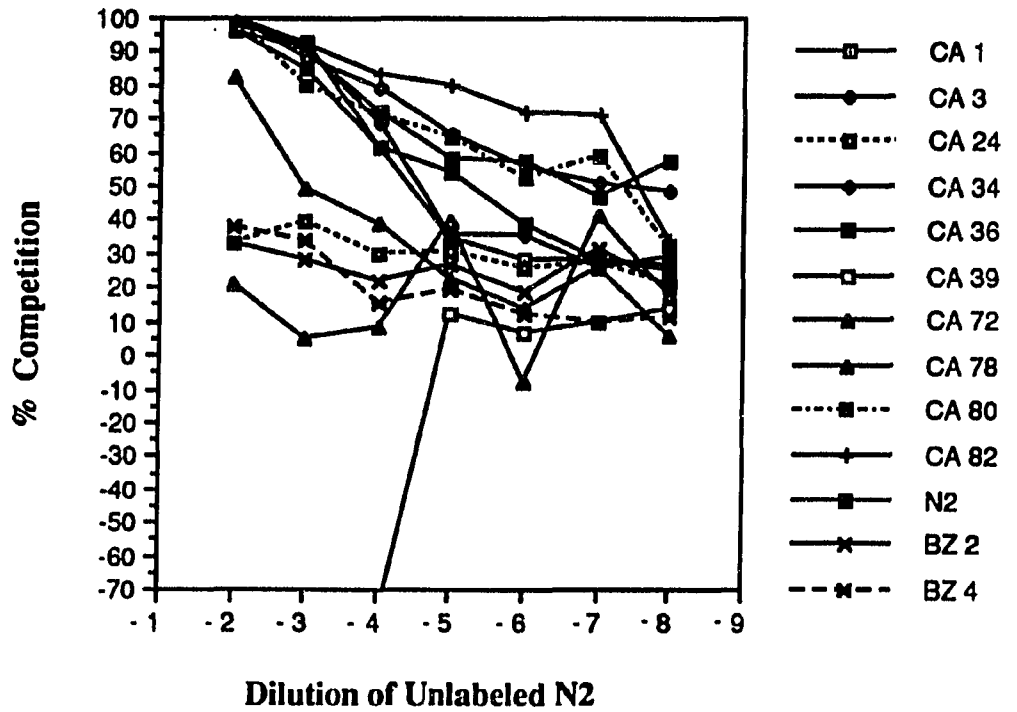
-9
Dilution of Unlabeled CA 82
CA 1
CA 3
CA 24
CA 34
CA 36
CA 39
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4

Figure 17: Simultaneous competitive binding assays of BVBV-7443 using N2 and BZ-2. Dilutions of labeled purified Mab (50% binding concentration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.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-4
-5
-6
-7
-8
-9
Dilution of Unlabeled N2
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CA 72
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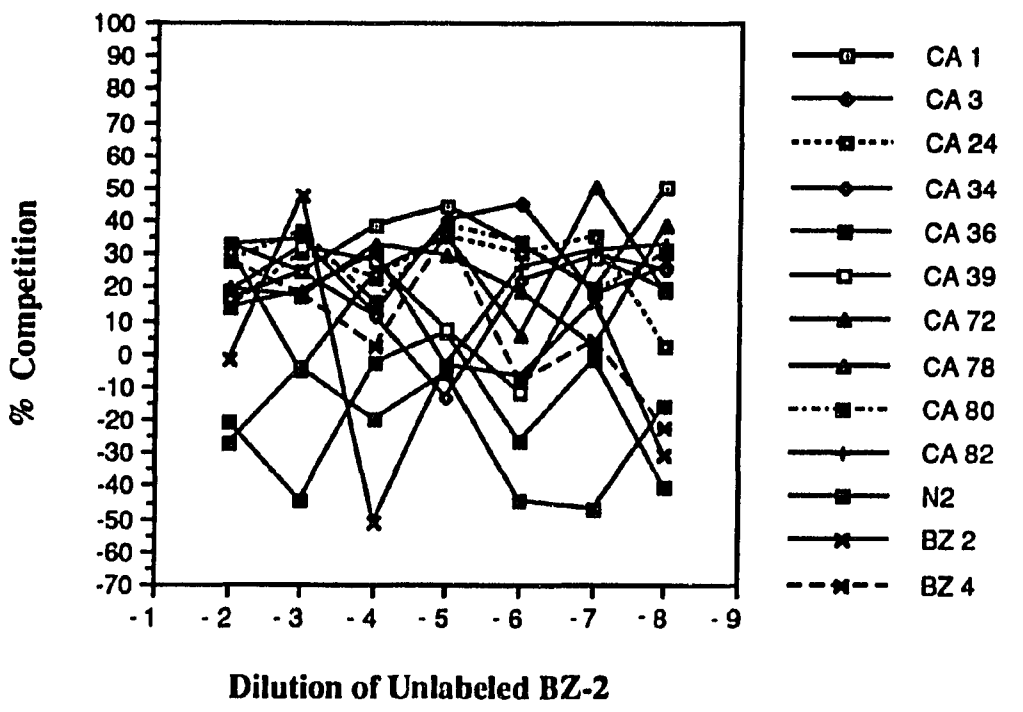
CA 80
CA 82
N2
BZ 2
BZ 4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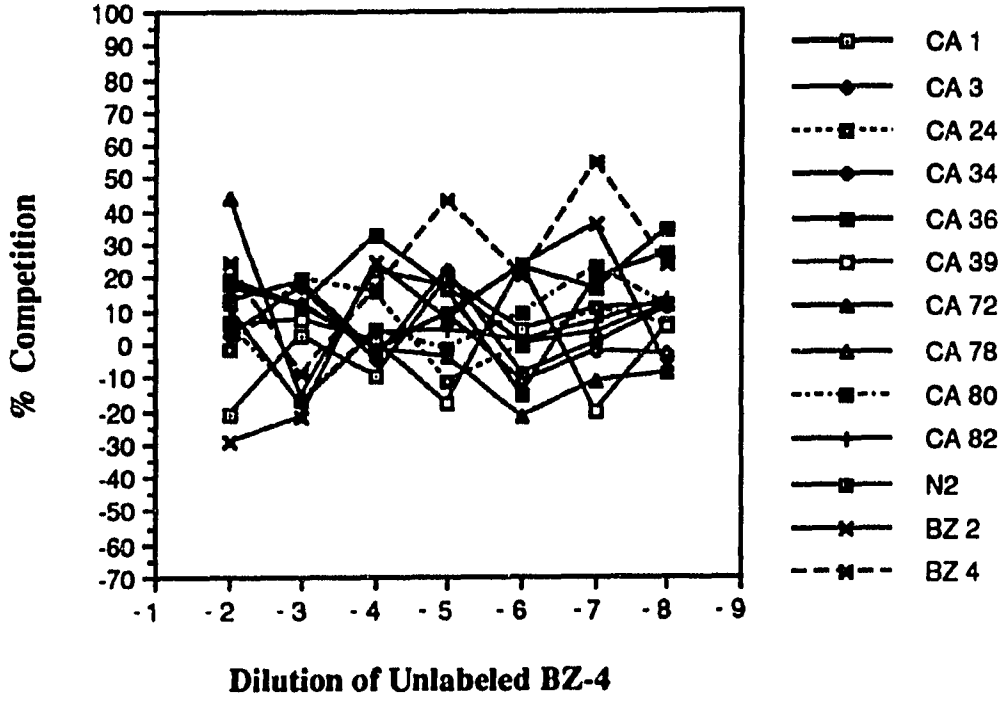
-9
Dilution of Unlabeled BZ-2
CA 1
CA 3
CA 24
CA 34
CA 36
CA 39
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4

Figure 18: Simultaneous competitive binding assays of BVBV-7443 using BZ-4. Dilutions of labeled purified Mab (50% binding concentration) and nonlabeled competing Mab (diluted ten-fold) were mixed and added to treated, antigen coated PVC microtiter wells in triplicate and incubated 3 hr at room temperature. Wells were washed six times each with TNE/W and ddH_2O, cut out, and the amount of bound labeled Mab was quantitated.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-9
Dilution of Unlabeled BZ-4
CA 1
CA 3
CA 24
CA 34
CA 36
CA 39
CA 72
CA 78
CA 80
CA 82
N2
BZ 2
BZ 4

APPENDIX B: MATERIALS

Serum Free Dulbecco's MEM

Dulbecco's MEM (Grand Island Biological Co., Gibco, Grand Island, NY) supplemented to 10% with BVDV-free fetal bovine serum, with the following added:

Non-essential amino acids	25.0 ml/liter
vitamin B12	0.625 mg/liter
biotin	0.25 mg/liter
sodium selenite	0.05 mg/liter
insulin	5.0 mg/liter
transferrin	5.0 mg/liter
L-glutamine	50.0 mg/liter
adenine	7.5×10^{-5}M
aminopterin	8×10^{-7}M
thymidine	1.6×10^{-5}M

Protein G sepharose Column buffer pH 7.0

Na_2HPO_4	0.02M
NaH_2PO_4	0.02M

Section II: NOVEL AND RAPID METHOD FOR PRODUCTION OF BOVINE VIRAL DIARRHEA VIRUS ANTIGEN

Novel and Rapid Method for Production of Bovine Viral Diarrhea Virus Antigen For Enzyme Immunoassays

Brief Report

R. C. Unfer,[1] and S. R. Bolin,[2]

Manuscript prepared for publication in Archives of Virology

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[2]United States Department of Agriculture, Agricultural Research Service, National Animal Disease Center, Ames, IA

Summary. Bovine viral diarrhea virus (BVDV) antigen was produced by two methods of lysis and compared for use in enzyme immunoassays (EIA) for detection of antibodies against BVDV. In one method, BVDV antigen was purified from lysates of infected bovine turbinate (BT) cells using glycerol-potassium tartrate density gradients. In the second method, BVDV-infected BT cells were solubilized and viral antigen released using the zwitterionic detergent CHAPS. Detergent was removed by chromatography and antigen was ready for immediate use. The relative BVDV antigen yield was determined by EIA, and solubilized BVDV antigen could be detected at a sixteen-fold greater dilution than density gradient-purified viral protein. Detergent solubilized viral antigen was used in an EIA to detect antibody to BVDV in fetal bovine sera. BVDV antigen obtained from one 150 cm^2 cell culture flask supplied sufficient antigen to assay 150 samples of fetal bovine serum. Results from the EIA correlated closely to those obtained using virus neutralization tests.

Bovine viral diarrhea virus (BVDV) is a widespread and economically important viral pathogen of cattle. Serologic surveys indicate 60 to 90 percent of cattle in the United States have antibodies against BVDV (Kahrs et al., 1964; Bolin et al., 1985). The two biotypes of BVDV, noncytopathic and cytopathic, usually cause subclinical or mild disease (Malmquist, 1968). Infection of pregnant dams is common, and frequently results in transplacental infection of the fetus. The outcome of fetal infection is influenced by gestational age (Orban et al., 1983; Liess et al., 1984; McClurkin et al., 1984). Infection during the first six months of pregnancy may result in fetal resorption, abortion, mummification, congenital defects, or the birth of weak, undersized calves (Kendrick, 1971; Brown et al., 1973; 1974; 1975). Fetuses infected during the last trimester are able to mount an active immune response against BVDV (Brown et al., 1979).

Fetal bovine serum is widely used in cell culture because of its growth promoting capacity, low concentration of immunoglobulins, and availability. Antibody against BVDV in fetal bovine sera has been detected by several investigators (Kniazeff et al., 1967; Boone et al., 1972; and Rossi and Kiesel, 1974). When fetal calf serum contaminated with antibody against BVDV is included in medium used to propagate pestiviruses, the virus yield is reduced (Van Oirschot, 1983). Additionally, fetal bovine serum containing BVDV antibody would be undesirable for production of monoclonal antibodies by cell culture.

Several microplate enzyme-linked immunosorbent assays (ELISA) tests have been developed for detection of antibodies to BVDV in cattle sera. Two types of ELISA, competitve-blocking assays (Westenbrink et al., 1986; Junti et al., 1987; Katz and Hansen, 1987), and indirect assays (Howard et al., 1985; Chu et al., 1985; Bock et al., 1986; Liauw and Eugster, 1986; Justewicz et al., 1987; Chu et al., 1987; Caquineau et al., 1988; Durham and Hassard, 1990) have been described. However, difficulties in working with BVDV including extensive antigen production procedures and low antigen yields limit the use of the ELISA.

Monolayers of bovine turbinate (BT) cells were grown in 490 cm^2 roller bottles and 150 cm^2 cell culture flasks, using F-15 MEM (F-15 Minimum Essential Eagles Medium, Grand Island Biological Co. Grand Island, NY) supplemented with 10% fetal bovine sera. The fetal bovine sera was determined to be free of BVDV using viral isolation techniques, and free of antibodies against BVDV using viral neutralization and indirect immuno-peroxidase staining procedures (Ridpath et al., 1991). BT cells grown in cell culture flasks and roller bottles were both infected with the Singer isolate of cytopathic virus as described elsewhere (McClurkin et al., 1974).

Ten roller bottles of BT cells infected with the Singer isolate of cytopathic virus and ten roller bottles containing mock-infected BT cells were subjected to one freeze-thaw cycle.

Cells were removed and sonicated for 30 seconds. The preparations were centrifuged at 8,000 x g for 45 minutes using a Type 19 rotor (Beckman Instruments, Palo Alto, CA) at 4 C, and the supernatant was harvested. Viral and cell proteins were pelleted from the supernatent by ultracentrifugation at 112,000 x g for 4 hours at 4 C in a SW 28 rotor (Beckman). Pelleted material was resuspended in minimal volumes of PBS (Dulbecco's phosphate buffered saline pH 7.2) and pooled. This material was layered onto 20% glycerol-60% potassium tartrate (w/v in PNE 0.1M Pipes, 0.1M NaCl, 5mM EDTA pH 7.4) linear gradients and centrifuged at 242,000 x g for 16 hours at 4 C in a VTi 50 rotor (Beckman). One milliliter fractions were collected, dialyzed against 0.001M phosphate buffer, and assayed for infective virus particles. Fractions containing infectious virus were pooled, pelleted by ultracentrifugation and resuspended in 100 ul of PBS. Mock-infected cellular protein was layered onto density-gradients, and fractionated. Fractions corresponding to virus-containing fractions were concentrated in the same manner. Protein concentrations were determined (Bio-Rad Protein Assay, Bio-Rad, Richmond, CA).

Solubilized BVDV antigen and mock-infected cellular antigen was produced from three 150 cm^2 cell culture flasks. Virus and cells were harvested when 90% of infected BT cell monolayers displayed cytopathic effects (vacuolation). The infected cell monolayers and mock-infected cell controls were washed once with PBS, and scraped off cell culture flasks. The cells were resuspended in minimal volumes of PBS (2 mls/flask) and solubilized with 13 mM CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate), Pierce Chemicals, Rockford, IL) for one hour on ice with stirring. Both preparations were sonicated (15 seconds), and cell material was sedimented by centrifugation for 15 minutes at 12,000 x g (Beckman Eppendorf centrifuge, Beckman). CHAPS was removed from the solubilized cell material by chromatography (Extracti-Gel D detergent removal columns, Pierce) using PBS as column buffer. Detergent-solubilized BVDV antigen had a final

volume of 2 ml/flask. Protein concentrations of virus and mock-infected preparations were determined as before.

Viral antigen prepared by either density-gradient purification or detergent solubilization was titrated. Two-fold serial dilutions of BVDV and uninfected BT-cell control antigen were made in TNE (0.05M Tris-HCL, 0.15M NaCl, 5mM EDTA) and coated onto 96-well Polyvinyl chloride (PVC) microtitration plates (Dynatech Laboratories, Chantilly, VA) by drying overnight at 37 C. Following a 5 minute methanol fixation step (Frankel and Gerhard, 1979), microtitration plate wells were washed three times with 300 ul of TNE/W (0.05% Tween-20). The microtitration plates were drained, and blotted on absorbent paper. Nonspecific binding sites were blocked by adding 100 ul of blocking buffer (PBS, 0.5% fish gelatin, Sigma Chemicals, St. Louis, MO) (Saravis, 1984) in to each well, and the plates were then incubated one hour at room temperature. Plates were washed once with TNE/W, and antibody-positive bovine serum diluted 1:100 in ELISA buffer (TNE, 2.0% Tween-20, 0.5% fish gelatin, 0.78M NaCl, 80 U/ml heparin) was added to all microtitration plate wells. Following a 30 minute incubation at 4 C, microtitration plate wells were washed 5 times with TNE/W, and 50 ul of protein G-peroxidase conjugate (Zymed Laboratories, San Francisco, CA) diluted 1:8000 in PBS was added to each well for a 30 minute incubation at 4 C. Microtitration plates were washed five times with TNE/W, and 100 ul of ABTS (Kirkegaard and Perry, Gaithersburg, MD) was added to each well and incubated at room temperature. The reaction was stopped by addition of 50 ul of a 1:400 dilution of HF (hydrogen fluoride) to each well, and the optical density (OD) at 410 nm of each well was determined using an ELISA reader (Dynatech).

Each test consisted of a three well series; one preabsorbtion well, one negative control well, and one test well. Initially, 50 ul of a 450 ug/ml uninfected cell antigen suspension was added to each preabsorbtion and control well, and 50 ul of an equal concentration of viral

antigen was added to each test well. After an overnight incubation at 37 C, the dried wells were fixed with methanol for 5 minutes, then washed three times with TNE/W. Next, 100 ul of blocking buffer was added to each well and incubated one hour at room temperature. Plates were washed once with TNE/W, and 20 ul of undiluted fetal bovine serum was added to the preabsorbtion wells. After a one hour incubation at room temperature, 5 ul of pre-absorbed fetal bovine serum was transfered to control and test wells, and 45 ul of ELISA buffer was added. The microtitration plates were incubated 30 minutes at 4 C, and then washed five times with TNE/W. Next, 50 ul of Protein G-Peroxidase conjugate (Zymed) diluted 1:8000 in PBS was added to each well, and the plates were incubated 30 minutes at 4 C. Microtitration plates were washed five times with TNE/W, and 100 ul of ABTS (Kirkegaard and Perry) was added to each well and incubated at room temperature for 30 minutes. The reaction was stopped as before, and the optical density (OD) at 410 nm of each microtitration plate well was obtained. Data were analyzed using the Softmax ELISA program (Molecular Devices, Menlo Park, CA). The reactivity of the fetal bovine sera was expressed in EIA units defined as:

$$\frac{\text{(OD of test well) - (OD of cell control well)}}{\text{(mean net OD of positive control) - (mean net OD of negative control)}} \times 100$$

Fetal bovine sera that were positive (82 samples) or negative (209 samples) for virus neutralizing antibodies by immunoperoxidase staining as described elsewhere (Ridpath et al., 1991), and virus neutralization test (Carbrey et al., 1974), were collected and stored at -20 C. The samples of sera were from pools of serum obtained from two to three fetuses.

Positive control serum was obtained from a cow vaccinated with modified-live virus vaccine. Negative control serum was obtained from a cow that lacked detectable antibodies

against BVDV using radioimmunoprecipitation, serum neutralization, and indirect immunoperoxidase staining.

Ten roller bottles of BVDV-infected BT cells yielded 0.4 ng of protein, from pooled and pelleted fractions of glycerol-potassium tartrate gradients, as measured by protein assay. Each 150 cm^2 cell culture flask solubilized with CHAPS detergent yielded an average of 1.2 mg of a mixture of viral and BT cell protein. When titrated against bovine serum containing antibody against BVDV in an EIA, density-gradient purified viral protein was not detected beyond the 1:4 dilution. In the detergent-solubilized infected cell lysate, viral antigen was titrated to the 1:64 dilution.

Detergent-solubilized viral antigen and uninfected BT cell antigen were coated onto microtiter plates and used in an EIA to detect antibodies against BVDV in fetal bovine serum. Reactivity of the fetal bovine serum was expressed in EIA units. Results are listed in table 1. A value of ten EIA units was chosen as the cut-off between positive and negative results. Of 82 viral neutralization positive samples of fetal bovine serum, the ELISA identified 10 as negative. Of these 10 serum lots, 5 had a viral neutralization titer of 2. A total of 200 viral neutralization negative lots were tested by EIA, and 6 tested positive. The EIA in this study had a relative sensitivity of 87.8%, and a relative specificity of 97.0%.

Table 1. Comparison of enzyme immunoassay with serum neutralization for detection of anti-BVDV antibodies.

EIA result	Serum neutralization test result	
	Positive[1]	Negative
Positive[2]	72 (a)	6 (b)
Negative	10 (c)	194 (d)

[1] Titer equal to or greater than 1/2
[2] Activity in ELISA units greater than 10
Relative sensitivity (a/a+c)=87.8%
Relative specificity (d/b+d)=97.0%
Predictive value (a/a+b)+92.3%

This study presents a novel and rapid method for production of BVDV antigen for use in an EIA. Previously described methods for BVDV antigen production involved multiple centrifugation steps and purification through sucrose or glycerol-potassium tartrate density gradients (Chu et al., 1987; Magar and Lecomte, 1987). Such purification procedures cannot be completed rapidly, and the end result is a low yield of BVDV antigen.

In this study, infected monolayers of BT cells were solubilized with the zwitterionic detergent CHAPS to release native virus particles and viral proteins. This method of antigen production is much more rapid than density-gradient purification. Preparation of detergent-solubilized BVDV antigen is completed less than 24 hours following infection of cell monolayers, while at least six days are necessary to produce density-gradient purified BVDV antigen. In addition, rather than propagating BVDV in roller bottles in order to maximize antigen yield, cell culture flasks are used, eliminating much of the media and supplies necessary for BVDV antigen production. Since volumes during each step of the procedure were purposely kept low, density-gradients were not needed, nor were multiple centrifugation steps and pelleting of virus necessary. This eliminated most of the steps during which the antigen could be lost or denatured. Following detergent removal by chromatography, virus protein suspensions were ready for immediate use in an EIA. Although equal starting concentrations of antigen were used and diluted identically, the detergent-solubilized BVDV antigen could be diluted sixteen times further than gradient purified viral antigen. Repeated manipulations during the production of gradient purified BVDV antigen probably contributed to the low yield. In addition, since BVDV forms broad bands in density gradients, fractions containing BVDV protein can only be identified with assays that detect infectious virus particles. Any disrupted virus or viral proteins would be lost and unavailable for immunoassays.

The choice of a suitable detergent for purification of membrane proteins is usually based upon the ability of the detergent to preserve native properties of the protein, especially important is antigenicity. Detergents can be denaturing or nondenaturing. Anionic detergents such as sodium dodecyl sulfate (SDS) or cationic detergents completely disrupt membranes and denature proteins. Nondenaturing detergents can be divided into nonionic detergents such as Triton X-100, and zwitterionic detergents such as CHAPS (Bitonti et al., 1982; Crawford et al., 1984; Mukhlis et al., 1986). Two other important considerations when choosing a solubilizing agent are, the degree of artifactual aggregation to form nonspecific protein complexes, and ease of removal of the detergent from solubilized proteins. Nonionic detergents are not efficient at disaggregating solubilized proteins, while zwitterionic detergents are efficient at disrupting protein-protein interactions, and have a high CMC (critical micelle concentration) which allows the detergent to be removed easily by dialysis. Previous studies have already described the release of viral glycoproteins via zwitterionic solubilization (Crawford et al., 1984).

Detection of antibodies to BVDV in fetal bovine serum is currently performed by indirect fluorescent antibody tests, immunoperoxidase staining, or by serum neutralization tests. Each assay is not without its disadvantages, such as nonspecificity, ambiguous results, and the time necessary to set up and perform the assays. ELISA's have been adapted for use with BVDV to detect antibodies to BVDV in cattle sera, but these assays require extensive antigen production protocols (Howard et al., 1985; Chu et al., 1985; Katz and Hanson, 1987). The antigen production method introduced here is fast and simple. Whereas purification procedures would involve as many as six days (from infection of cell cultures to coating of microtiter plates) to prepare BVDV antigen , detergent solubilized BVDV antigen is available less than twenty-four hours. This study has demonstrated that more

immunologically recognizable BVDV antigen can be produced by zwitterionic detergent solubilization in a shorter time than density gradient purification.

REFERENCES

Bitonti, A. J., G. Koski, and W. A. Kleen. 1982. Solubilization of membrane proteins by sulfobetaines, novel zwitterionic surfactants. Biochemistry 21:3650-3653.

Bock, R. E., G. W. Burgess, and I. C. Douglass. 1986. Development of an enzyme linked immuno-sorbent assay (ELISA) for the detection of bovine serum antibody to bovine viral diarrhea virus. Aust. Vet. J. 63(12):406-408.

Bolin, S. R., A. W. McClurkin, R. C. Cutlip, and M. F. Coria. 1985. Response of cattle persistently infected with noncytopathic bovine viral diarrhea virus to vaccination for bovine viral diarrhea and to subsequent challenge exposure with cytopathic bovine viral diarrhea virus. Am. J. Vet. Res. 46:2467-2470.

Boone, C. W., N. Mantel, T. D. Carup Jr., E. Kazam, and R. E. Stevenson. 1972. Quality control studies on fetal bovine serum used in tissue culture. In Vitro 7:174-189.

Brown, T. T., A. De Lahunta, F. W. Scott, R. F. Kahrs, K. McEntee, and J. M. Gillespie. 1973. Virus induced congenital anomalies of the bovine fetus. II. Histopathology of cerebellar degeneration (hypoplasia) induced by the virus of bovine viral diarrhea-mucosal disease. Cornell Vet. 63:561-578.

Brown, T. T., A. De Lahunta, S. I. Bistner, F. W. Scott, and K. McEntee. 1974. Pathogenic studies of infection of the bovine fetus with bovine viral diarrhea virus. I. Cerebellar atrophy. Vet. Pathol. 11:486-505.

Brown, T. T., A. De Lahunta, S. I. Bistner, F. W. Scott, and K. McEntee. 1975. Pathogenic studies of infection of the bovine fetus with bovine viral diarrhea virus. II. Ocular lesions. Vet. Pathol. 12:394-404.

Brown, T. T., R. D. Schultz, J. R. Duncan, and S. I. Bistner. 1979. Serological response of the bovine fetus to bovine viral diarrhea. Infection and Immunity 25:93-87.

Caquineau, L., A. Douart, and J. Lecoanet. 1988. Mise au point d'une Methode immunoenzymatique E.L.I.S.A. pour la detection des anticorps anti virus B.V.D. dans le serum des bovins. Rec. Med. Vet. 164(5):381-383.

Carbrey, E. A., D. R. Downing, M. L. Snyder, S. J. Wessman, and G. A. Gustafson. 1974. Microtiter and automated serologic techniques for diagnostic virology. Proc. U.S. Anim. Health Assoc. 77:553-562

Chu, H-J., Y. C. Zee, A. A. Ardans, and K. Dai. 1985. Enzyme-linked immunosorbent assay for the detection of antibodies to bovine viral diarrhea virus in bovine sera. Vet. Microbiol. 10:325-333.

Chu, H-J., M. M. Sawyer, C. A. Anderson, R. J. Higgins, and Y. C. Zee. 1987. Enzyme-linked immunosorbent assay for the detection of antibodies to bovine virus diarrhea virus in sera from border disease virus-infected sheep. Can. J. Vet. Res. 51:281-283.

Crawford, C. R., F. A. Mukhlis, R. Jennings, J. S. Oxford, D. J. Hockley, and C. W. Potter. 1984. Use of a zwitterionic detergent for the disruption of influenza virus. I. Preparation and characterization of disrupted virions. Vaccine 2:193-198.

Durham, P. J. K. and L. E. Hassard. 1990. An enzyme-linked immunosorbent assay (ELISA) for antibodies to bovine viral diarrhea virus. Vet. Microbiol. 22:1-10.

Frankel, M. E. and W. Gerhard. 1979. The rapid determination of binding constants for antiviral antibodies by a radioimmunoassay. An analysis of the interaction between hybridoma proteins and influenza virus. Mol. Immunol. 16:101-106.

Howard, C. J., M. C. Clarke, and J. Brownlie. 1985. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to bovine viral diarrhea virus (BVDV) in cattle sera. Vet. Microbiol. 10:359-369.

Junti, N., B. Larsson, and C. Fossum. 1987. The use of monoclonal antibodies in enzyme linked immunosorbent assays for detection of antibodies to bovine viral diarrhea virus. J. Vet. Med. B34:356-363.

Justewicz, D. M., R. Magar, G. Marsolais, and J. Lecomte. 1987. Bovine viral diarrhea virus-infected MDBK monolayer as antigen in enzyme-linked immunoassay (ELISA) for the measurement of antibodies in bovine sera. Vet. Immun. Immunopathol. 14:377-384.

Kahrs, R., G. Atkinson, and J. A. Baker. 1964. Serological studies on the incidence of bovine viral diarrhea, infectious bovine rhinotracheitis, bovine myxovirus parainfluenza-3, and Leptospira pomona in New York state. Cornell Vet. 54:360-369.

Katz, J. B., and S. K. Hanson. 1987. Competitive and blocking enzyme-linked immunoassay for the detection of fetal bovine serum antibodies to bovine viral diarrhea virus. J. Virol. Meth. 15:167-175.

Kendrick, J. W. 1971. Bovine viral diarrhea-mucosal disease virus infection of pregnant cows. Am. J. Vet. Res. 32:533-544.

Kniazeff, A. J., V. Rimea, and L. Gaeta. 1967. Gamma-globulin in foetal bovine serum: significance in virology. Nature 214:805-806.

Liauw, H., and A. K. Eugster. 1986. Enzyme-linked immunosorbent assay for the diagnosis of bovine viral diarrhea. Southwest. Vet. 37:47-50.

Liess, B., S. Orban, H. R. Frey, G. Trautwein, W. Wiefel, and H. Blindow. 1984. Studies on transplacental transmissibility of a bovine virus diarrhoea (BVD) vaccine virus in cattle. II. Inoculation of pregnant cows without detectable neutralizing antibodies to BVD virus 90-229 days before parturition (51st to 190th day of gestation). J. Vet. Med. B 31:669-681.

Magar, R., H. C. Minocha, and J. Lecomte. 1988. Bovine viral diarrhea virus proteins: Heterogeneity of cytopathogenic and non-cytopathogenic strains and evidence of a 53k Glycoprotein neutralization epitope. Vet. Microbiol. 16:303-314.

Malmquist, W. A. 1968. Bovine viral diarrhea-mucosal disease complex: etiology, pathogenesis, and applied immunity. J. Amer. Vet. Med. Assoc. 152:763-768.

McCurkin, A. W., E. C. Pirtle, and M. F. Coria. 1974. Comparison of low- and high-passage bovine turbinate cells for assay of bovine viral diarrhea virus. Archiv. ges. Virusforsch. 45:285-289.

McClurkin, A. W., E. T. Littledike, R. C. Cutlip, G. H. Frank, M. F. Coria, and S. R. Bolin. 1984. Production of cattle immunotolerant to bovine viral diarrhea virus. Can. J. Comp. Med. 48:156-161.

Mukhlis, F. A., R. Jennings, T. J. Stephenson, T. Quasim, and C. W. Potter. 1986. Characterization and immunogenicity of HSV-1 antigens obtained following zwitterionic detergent treatment. Vaccine 4:191-196.

Orban, S., B. Liess, S, M. Hafez, H. R. Frey, H. Blindow, and B. Sasse-Patzer. 1983. Studies on transplacental transmissibility of a bovine virus diarrhoea (BVD) vaccine virus. I. Inoculation of pregnant cows 15 to 90 days before parturition (190th to 265th day of gestation). J. Vet. Med. B 30:619-634.

Ridpath, J. F., T. L. Lewis, S. R. Bolin, and E. S. Berry. 1991. Antigenic and genomic comparison between non-cytopathic and cytopathic bovine viral diarrhoea viruses isolated form cattle that had spontaneous mucosal disease. J. Gen. Virol. 72:725-729.

Rossi, C. R., and G. K. Kiesel. 1971. Microtiter tests for detecting antibody in bovine serum to parainfluenza 3 virus, infectious bovine rhinotracheitis virus, and bovine virus diarrhea virus. Appl. Microbiol. 22:32-36.

Rossi C. R., and G. K. Kiesel. 1974. Antibody to viruses affection cattle in commercial tissue culture grade fetal calf serum. J. Appl. Microbiol. 27:114-117.

Saravis, C. A. 1984. Improved blocking of nonspecific antibody binding sites on nitrocellulose membranes. Electrophoresis 5:54-55.

Van Oirschot, J. T. 1983. Congenital infections with non-arbovirus Togaviruses. Vet. Microbiol. 8:321-361.

Westenbrink, F., G. J. Middel, P. J. Straver, and P. W. de Leeuw. 1986. A blocking enzyme-linked immunosorbent assay (ELISA) for bovine virus diarrhea virus serology. J. Vet. Med. B33:354-361.

GENERAL DISCUSSION AND SUMMARY

The first study utilized a panel of thirteen monoclonal antibodies to characterize the neutralizing epitopes on the 53 kd glycoprotein of BVDV. The physical nature of the epitopes, the relationship of these epitopes to serum complement, and their position on the glycoprotein relative to carbohydrate moieties, was examined. In addition, the topographic orientation of the epitopes on the envelope glycoprotein was determined by competitive binding analysis with the panel of monoclonal antibodies. Competition between pairs of antibodies may indicate the antibodies belong to the same binding group, i.e. recognize the same epitope. However, several factors may explain apparent competition between two antibodies. First, the physical bulk of a monoclonal antibody bound to an epitope may sterically hinder the binding of another antibody to a separate and distinct epitope. Secondly, the binding of one monoclonal antibody at a distinct epitope may induce a conformational change in the protein, masking the epitope of another antibody. Third, the labeling of a monoclonal antibody may alter its ability to bind to its specific epitope (Yewdell and Gerhard, 1981). Conversely, while failure of competition provides evidence that the antibodies bind to distinct sites on the antigen, antibodies will not compete with each other for the same epitope if they possess significantly different affinities for the epitope.

Data suggest the epitope recognized by each monoclonal antibody was conformationally-dependent, or was a discontinuous epitope formed by amino acid residues that were not contiguous in the primary sequence. The neutralization titers of each monoclonal antibody in the presence and absence of serum complement, gave an indication of the relative position of epitopes on the viral glycoprotein. Since none of the monoclonal antibodies was dependent upon the presence of serum complement for neutralization, the epitope for each antibody may be located at the distal end of the glycoprotein, away from the

surface of viral envelope. These results correlate with those determined following endoglycosidase treatment of the viral glycoprotein. Treatment of BVDV glycoproteins indicated that the neutralizing epitopes on gp 53 of BVDV were either located in the portion of the glycoprotein that carried the carbohydrate moieties, or were in close proximity to this region. The removal of the carbohydrate moieties likely altered the conformation of the glycoprotein epitopes in that region, and affected the binding of antibodies.

Two potential antigenic domains were identified on the BVDV glycoprotein. Eleven of the monoclonal antibodies bound to overlapping epitopes in one antigenic domain, while another domain was recognized by two monoclonal antibodies, Ca 24 and Ca 78. However, since this study did not use an isolate to which both Ca 24 and 78 bound, their epitopes could not be distinguished.

Van Drunen et al. (1985) determined that the extent of blocking in a competitive binding assay is directly proportional to the avidity of each monoclonal antibody. The relative avidity of each monoclonal antibody was determined for both isolates of BVDV used in this study. This was used to estimate the amount of labeled antibody necessary for each assay. The binding constant of each monoclonal antibody was also determined, since this would provide more information on whether any competition between two antibodies was true competition or an artifact of differences in binding strength. The binding constants for three monoclonal antibodies in this panel (table 2) varied significantly from the others. But the remainder of the panel had similar binding strengths.

The second study described a novel and rapid protocol for BVDV antigen production using the zwitterionic detergent CHAPS to solubilize infected cell monolayers and obtain antigen without the need for time-consuming purification steps. The study compared the ease of density gradient purification and detergent solubilization, as well as the yield of viral antigen from the two methods, and determined that a greater amount of BVDV antigen could

be obtained in less time than the standard antigen purification procedure. While density-gradient purification procedures would involve as many as six days to prepare BVDV antigen, detergent-solubilized BVDV antigen is available less than twenty-four hours following infection. Use of the zwitterionic detergent CHAPS ensured that protein was not denatured as could be the case when anionic detergents are used for solubilization. Detergent could be rapidly removed from protein suspensions by chromatography, or removed by overnight dialysis. Most of the contaminating cell protein was removed by a centrifugation step, and the viral antigen was ready for immediate use.

The utility of the viral antigen was tested in an enzyme immunoassay. Fetal bovine sera was assayed for presence of antibody against BVDV, and results were compared to those determined by a viral neutralization test. This enzyme immunoassay used several steps to reduce inherent high background associated with BVDV serological assays. Some inert blocking proteins commonly used used to prevent nonspecific binding by antibodies (bovine serum albumen, and skim milk) are not acceptable due to the potential presence of BVDV antibody. This difficulty was overcome by using a fish gelatin blocking agent (Saravis, 1984). Fish gelatin should be an immunologically unfamiliar protein to cattle, with little likelihood that the bovine immune system would produce antibodies to it. A methanol fixation step (Frankel and Gerhard, 1979), a buffer that contained high salt and Tween-20 concentrations, and heparin (Dietzgen and Francki, 1987), each contributed to a reduction in non-specific background. Heparin is a cationic protein that binds to highly anionic molecules, i.e. proteins or antibodies, and also prevents nonspecific binding. In addition, the protein G-peroxidase conjugate eliminated the need for a second antibody species, and may also have helped to reduce the nonspecific background. A high correlation between the enzyme immunoassay and virus neutralization test was seen. This study presents a rapid method for generation of BVDV antigen that can be used for easy detection of BVDV

antibodies in fetal bovine serum, and can be adapted to detection of BVDV antibodies in vaccinated animals.

In conclusion, the work presented here described the antigenic characterization of the 53 kd envelope glycoprotein of BVDV, and a novel and rapid method for production of BVDV antigen using a zwitterionic detergent, that can be used in enzyme immunoassays as well as the competitive binding assays described in the first study.

LITERATURE CITED

Akkina, R. K. 1982. Analysis of immunogenic determinants of bovine viral diarrhea virus. Ph.D thesis, University of Minnesota, MN.

Atluro, D., W. Notowidjojo, D. W. Johnson, and C. C. Muscoplat. 1979. Suppression of vitro immunoglobulin biosynthesis in bovine spleen cells by bovine viral diarrhoea virus. Clin. Immun. Immunopathol. 13:254-260.

Avellini, G., G. Castrucci, B. Morettini, and V. Chilli. 1968. Bovine Viral Diarrhea in Italy. II. Reproduction of the disease. Archiv. ges Virusforsch. 24:65-75.

Aynaud, J. M., C. Rigaud, Y. Le Turdu, C. Galicher, J. Lombard, G. Corthier, and H. Laude. 1974. Peste porcinee classique; les variations serologiques du virus en France et leur role dans l'evolution de la maladie sous sa forme subclinique ou chronique sur le terrain. Annal. de Rech. Vet. 5:57-85.

Avrameas, S., and J. Uriel. 1966. Methode de marquage d'antigenes et d'anticorps avec des enzymes et son application en immunodifusion. C. R. Acad. Sci. Paris 262:2543-2561.

Baker, J. C. 1987. Bovine viral diarrhea virus: A review. J. Am. Vet. Med. Assoc. 190:1449-1458.

Baker, J. A., G. J. York, J. H. Gillespie, and G. B. Mitchell. 1954. Virus Diarrhea in Cattle. Am. J. Vet. Res. 15:525-531.

Barber, D. M. L., P. F. Nettleton, and J. A. Herring. 1985. Disease in dairy herd associated with the introduction and spread of bovine virus diarrhea virus. Vet. Res. 117:459-464.

Barlow, R. M., P. F. Nettleton, A. C. Gardiner, A. Greig, J. R. Cambell, and J. M. Bonn. 1986. Persistent bovine virus diarrhoea virus infection in a bull. Vet. Rec. 118:321-324.

Bielefedt-Ohmann, H., and B. Bloch. 1982. Electron microscopic studies of bovine viral diarrhea virus in tissues of diseased calves and in cell cultures. Arch. Virol. 71:57-74.

Bielefedt-Ohmann, H., B. Bloch, W. C. Davis, and J. Askaa. 1988. Bovine viral diarrhea virus infection in peripheral blood mononuclear cells from persistently viraemic calves studied by correlative immunoelectron microscopy. J. Vet. Med. B 35:477-482.

Binkhorst, G. J., D. L. H. Journee, W. Wouda, P. J. Straver, and J. H. Vos. 1983. Neurological disorders, virus persistence, and hypomyelination in calves due to intrauterine infection with bovine viral diarrhoea virus. Veterinary Quarterly 5:145-150.

Bock, R. E., G. W. Burgess, and I. C. Douglass. 1986. Development of an enzyme-linked immunosorbent assay (ELISA) for the detection of bovine serum antibody to bovine viral diarrhea virus. Aust. Vet. J. 63:405-408.

Boere, W. A., T. Harmsen, J. Vinje, B. J. Benaissa-Trouw, G. A. Kraayeveld, and H. Snipe. 1984. Identification of distinct antigenic determinants on Semliki Forest virus by using monoclonal antibodies with different antiviral activities. J. Virol. 52(2):575-582.

Bolin, S. R., A. W. McClurkin, and M. F. Coria. 1985a. Effects of bovine viral diarrhea virus of percentages of absolute numbers of circulating B and T lymphocytes in cattle. Am. J. Vet. Res. 46:884-886.

Bolin, S. R., A. W. McClurkin, and M. F. Coria. 1985b. Frequency of persistent bovine viral diarrhea virus infection in selected herds. Am. J. Vet. Res. 46:2385-2387.

Bolin, S. R., A. W. McClurkin, R. C. Cutlip, and M. F. Coria. 1985c. Severe clinical disease induced in cattle persistently infected with noncytopathic bovine viral diarrhea virus by superinfection with cytopathic bovine viral diarrhea virus. Am. J. Vet. Res. 46:573-576.

Bolin, S. R., A. W. McClurkin, R. C. Cutlip, and M. F. Coria. 1985d. Response of cattle persistently infected with noncytopathic bovine viral diarrhea virus to vaccination for bovine viral diarrhea and to subsequent challenge exposure with cytopathic bovine viral diarrhea virus. Am. J. Vet. Res. 46:2467-2470.

Bolin, S. R., J. A. Roth, E. K. Uhlenhopp, and J. F. Pohlenz. 1987. Immunologic and serologic findings in a bull chronically infected with noncytopathic bovine viral diarrhea virus. J. Am. Vet. Med. Assoc. 190:1015-1017.

Bolin, S. , V. Moennig, N. E. Kelso Gourley, and J. Ridpath. 1988. Monoclonal antibodies with neutralizing activity segregate isolates of bovine viral diarrhea virus into groups. Arch. Virol. 99:117-123.

Brown, T. T., A. De Lahunta, S. I. Bistner, F. W. Scott, and K. McEntee. 1974. Pathogenic studies of infection of the bovine fetus. I: Cerebellar atrophy. Vet. Pathol. 11:486-505.

Brownlie, J. 1975. Clinical aspects of bovine viral diarrhea/mucosal disease complex in cattle. In Pract. 7:195:202.

Brownlie, J., M. C. Clarke, and C. J. Howard. 1984. Experimental production of fatal mucosal disease in cattle. Vet. Rec. 114:535-537.

Brownlie, J., M. C. Clarke, C. J. Howard, and D. H. Pocock. 1987. Pathogenesis and epidemiology of bovine virus diarrhea virus infection of cattle. Ann. Rech. Vet. 18:157-166.

Cammack, N., and E. A. Gould. 1986. Topographical analysis of epitope relationships on the envelope glycoprotein of Yellow Fever 17D vaccine and the wild type Asibi parent virus. Virology 150:333-341.

Caquineau, L., A. Douart, and J. Lecoanet. 1986. Restatement of an immuno-enzymatic method. (ELISA) to detect antibodies to bovine viral diarrhea virus in bovine sera. Rec. Med. Vet. 164:381-383.

Carbrey, E. A. 1988. Diagnostic procedures. Pp. 99-114. B. Liess, ed. In Classical Swine Fever and Related Diseases. Martinus Nijhoff, The Hague.

Carlson, R. G., W. R. Pritchard, and L. P. Doyle. 1957. The pathology of virus diarrhea of cattle in Indiana. Am. J. Vet. Res. 18:560-568.

Casaro, A. P. E., J. W. Kendrick, and P. Kennedy. 1971. Response of the bovine fetus to bovine viral diarrhea-mucosal disease virus. Am. J. Vet. Res. 32:1543-1561.

Castrucci, G., V. Cilli, and G. Gagliardi. 1967. Bovine viral diarrhea in Italy I: Isolation and characterization of the virus. Archiv. ges. Virusforsch. 24:48-64.

Cay, B., G. Chappuis, Z. Dinter, S. Edwards, I. Greiser-Wilke, M. Gunn, P. Have, G. Hess, N. Junti, B. Liess, A. Mateo, P. McHugh, V. Moennig, P. Nettlton, and G. Wensvoort. 1989. Comparative analysis of monoclonal antibodies against pestiviruses: Report of an international workshop. Vet. Microbiol. 20:123-129.

Cecilia, J. E., D. A. Gadkari, N. Kedarnath, and S. V. Ghosh. 1988. Epitope mapping of Japanese encephalitis virus envelope protein using monoclonal antibodies against an Indian strain. J. Gen. Virol. 69:2741-2747.

Chasey, D., and P. L. Roeder. 1981. Virus-like particles in bovine turbinate cells infected with Bovine Viral Diarrhea/Mucosal Disease virus. Arch. Virol. 67:325-332.

Chu, H.-J., Y. C. Zee, A. A. Ardans, and K. Dai. 1985. Enzyme-linked Immuno-sorbent Assay for the detection of antibodies to bovine viral diarrhea virus in bovine sera. Vet. Microbiol. 10:325-333.

Chu, H.-J., M. M. Sawyer, C. A. Anderson, R. J. Higgins, and Y. C. Zee. 1987. Enzyme-linked Immunosorbent Assay for the detection of antibodies to Bovine viral diarrhea virus in sera from Border disease virus-infected sheep. Can. J. Vet. Res. 51:281-283.

Collett, M. S., R. Larson, C. Gold, D. Strick, D. K. Anderson, and A. F. Purchio, A. F. 1988a. Molecular cloning and nucleotide sequence of pestivirus bovine viral diarrhea virus. Virology 165:191-199.

Collett, M. S., R. Larson, S. K. Belzer, and E. Retzel. 1988b. Proteins encoded by bovine viral diarrhea virus: the genomic organization of a pestivirus. Virology 165:200-208.

Corapi, W. V., R. O. Donis, and E. J. Dubovi. 1988. Monoclonal antibody analyses of cytopathic and noncytopathic viruses from fatal bovine viral diarrhea virus infections. J. Virol. 62:2823-2827.

Coria, M. F., and A. W. McClurkin. 1978. Specific immune tolerance in an apparently healthy bull persistently infected with bovine viral diarrhea. J. Am. Vet. Med. Assoc. 172:449-451.

Coria, M. F., M. J. F. Schmerr, and A. W. McClurkin. 1983. Characterization of the major structural proteins of purified bovine viral diarrhea virus. Arch. Virol. 76:335-339.

Dale, B., R. T. White, and B. L. Cordell. 1987. Bovine viral diarrhea and hog cholera vaccines. European Patent Application number 87103218, Publication number 0236977, 16 September 1987.

Darbyshire, J. H. 1960. A serological relationship between swine fever and mucosal disease of cattle. Vet. Rec. 72:331.

Darbyshire, J. H. 1962. Agar gel diffusion studies with a mucosal disease of cattle. III. A serological relationship between a mucosal disease and swine fever virus. Res. Vet. Sci. 3:125-128.

Diderholm, H., and Z. Dinter. 1966. Interference between strains of Bovine Viral Diarrhea Virus and the capacity to suppress Interferon of a heterologous virus. Proc. Soc. Exp. Biol. Med. 121:976-980.

Diderholm, H., B. Hullseth, and Z. Dinter. 1973. Inhibition of hog cholera virus by acriflavine. Archiv. ges. Virusforsh. 42:300-302.

Dietzgen, R. G., and R. I. B. Francki. 1987. Nonspecific binding of immunoglobulins to coat proteins of certain plant viruses in immunoblots and indirect ELISA. J. Virol. Meth. 15:159-164.

Dinter, Z., and H. Diderholm. 1971. Bovine virus diarrhoea virus: Inhibition of growth by proflavine sulphate. Archiv. ges Virusforsh. 34:388-390.

Ditchfield, H., and E. D. Doane. 1964. Properties and classification of bovine viral diarrhea virus. Can. J. Comp. Med. 28:148-152.

Dittmar, D., T. J. Cleary, and A. Castro. 1979. Immunoglobulin G and M specific enzyme-linked immunosorbent assay for detection of dengue antibodies. J. Clin. Microbiol. 9:498-502.

Done, J. T., S. Terlecki, C. Richardson, J. W. Harkness, J. J. Sands, D. S. Patterson, D. Sweasy, I. G. Shaw, C. E. Winkler, and S. J. Duffell. 1980. Bovine virus diarrhoea-mucosal disease virus pathogenicity for the fetal calf following maternal infection. Vet. Rec. 106:473-479.

Donis, R. O., and E. J. Dubovi. 1987a. Differences in virus-induced polypeptides in cells infected by cytopathic and noncytopathic biotypes of bovine virus diarrhea- mucosal disease virus. Virology 158:168-173.

Donis, R. O., and E. J. Dubovi. 1987b. Glycoproteins of bovine viral diarrhoea virus-mucosal disease virus in infected bovine cells. J. Gen. Virol. 68:1607-1616.

Donis, R. O., W. Corapi, and E. J. Dubovi. 1988. Neutralizing monoclonal antibodies to bovine viral diarrhoea virus bind to the 56K to 58K protein. J. Gen Virol. 69:77-86.

Edwards, S., L. Wood, C. Hewith-Taylor, and T. W. Drew. 1986. Evidence for an immunocompromising effect of bovine pestivirus on bovid herpesvirus 1 vaccination. Vet. Res. Comm. 10:297-302.

Engvall, E., and P. Perlman. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8:871-875.

Engvall, E., and P. Perlman. 1972. Enzyme-linked immunosorbent assay ELISA. III: Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen coated tubes. J. Immunol. 109:129-136.

Fernelius, A. L. 1964. Noncytopathogenic bovine viral diarrhea viruses detected and titrated by immunofluorescence. Can. J. Comp. Microbiol. 28:121-126.

Fernelius, A. L. 1968. Characterization of bovine viral diarrhea viruses I: Determination of buoyant density. Archiv. ges Virusforsch. 25:211-218.

Frazier, C. L., and R. E. Shope. 1979. Detection of antibodies to alphavirus by enzyme-linked immunosorbent assay. J. Clin. Microbiol. 10:583-585.

Gillespie, J. H., and J. Baker. 1959. Studies on Virus Diarrhea. Cornell Vet. 49:439-443.

Gillespie, J. H., J. A. Baker, and K. McEntee. 1960. A cytopathogenic strain of Virus Diarrhea Virus. Cornell Vet. 50:73-79.

Gillespie, J. H., J. H. Gill, L. Coggins, J. Thompson, and J. A. Baker. 1961. Comparison neutralization tests of strains of virus isolated from Virus Diarrhea and Mucosal Disease. Cornell Vet. 51:155-159.

Gillespie, J. H., S. H. Madin, and N. B. Darby. 1962. Cellular resistance in tissue culture, induced by non-cytopathogenic strains, to a cytopathogenic strain of virus diarrhea virus of cattle. Proc. Exp. Biol. Med. 110:248-250.

Gillespie, J. H., S. H. Madin, and N. B. Darby Jr. 1963. Studies on virus diarrhea virus of cattle with special reference to its growth in tissue culture and its ether susceptibility. Cornell Vet. 53:276-282.

Graham, E. R. B., and A. Gottschalk. 1960. Studies on mucoproteins I: The structure of the prosthetic group of ovine submaxillary gland mucoprotein. Biochim. et. Biopys. Acta. 38:513-521.

Gratzek, J. B., P. P. Chennekatu, and R. F. Ramsey. 1966. Isolation and characterization of a strain of Infectious Bovine Rhinotracheitis Virus associated with enteritis in cattle: Isolation, serologic characterization, and induction of the experimental disease. Am. J. Vet. Res. 27:1567-1572.

Gratzek, J. B., R. F. Rosenbusch, G. M. Buenning. 1967. Plaque characteristics of four classes of bovine viruses. Am. J. Vet. Res. 28:137:140.

Gravell, M., P. H. Dorsett, O. Gutenson, and A. C. Ley. 1977. Detection of antibody to Rubella virus by Enzyme-Linked immunosorbent assay. J. Infect. Dis. S136:300-304.

Gray, E. W., and P. F. Nettleton. 1987. The ultrastructure of cell cultures infected with Border Disease and Bovine Viral Disease viruses. J. Gen. Virol. 68:2339-2346.

Guirakhoo, F., F. X. Heinz, and C. Kunz. 1989. Epitope model of Tick-borne encephalitis virus envelope glycoprotein E: Analysis of structural properties, role of carbohydrate side chain, and conformation changes occurring at acid pH. Virology 169:90-99.

Gutekunst, D. E., and W. A. Malmquist. 1963. Separation of a soluble antigen and infectious particles of bovine viral diarrhea viruses and their relationship to hog cholera. Can J. Comp. Med. 27:121-123.

Gutekunst, D. E., and W. A. Malmquist. 1964. Complement-fixing and neutralizing antibody response to bovine viral diarrhea and hog cholera antigens. Can. J. Comp. Med. 28:19-23.

Hafez, S. M., K. Petzoldt, and E. Reczko. 1968. Morphology of bovine viral diarrhea virus. Acta Virol. 12:471-473.

Harkness, J. W., J. J. Sands, and M. S. Richards. 1978. Serological studies of Mucosal Disease in England and Wales. Res. Vet. Sci. 24:98-103.

Heinz, F. X., R. Berger, W. Tuma, and C. Kunz. 1983. A topological and functional model of epitopes on the structural glycoprotein of Tick-borne encephalitis virus defined by monoclonal antibodies. Virology 126:525-537.

Heinz, F. X., C. Mandl, R. Berger, W. Tuma, and C. Kunz. 1984. Antibody-induced conformational changes resulting in enhanced avidity of antibodies to different antigenic sites on Tick-borne encephalitis glycoproteins. Virology 133:25-34.

Henchal, E. A., J. M. McCown, D. S. Burke, M. C. Seguin, and W. E. Brandt. 1985. Epitopic analysis of antigenic determinants on the surface of dengue-2 virions using monoclonal antibodies. Am. J. Vet. Trop. Med. 34:162-169.

Hermodsson, S., and Z. Dinter. 1962. Properties of bovine viral diarrhea virus. Nature 194:893-894.

Heuschele, W.P. 1978. New perspectives on the epidemiology of Bovine Viral Diarrhea - Mucosal Disease (BVD). Bov. Pract. 12:51-53.

Hofmann, H., W. Frisch-Niggemeyer, and F. Heinz. 1979. Rapid diagnosis of tick-borne encephalitis by means of enzyme linked immunosorbent assay. J. Gen. Virol. 42(3):505-511.

Horzinek, M. C. 1981. Nonarbo togavirus infections of animals: comparative aspects and diagnosis. Pp.442-478. E. Kurstak and C. Kurstak, eds. In Comparative Diagnosis of Viral Diseases. Academic Press, New York.

Horzinek, M. C., J. Maess, and R. Laufs. 1971. Studies on the substructure of Togaviruses. Arch. ges. Virusforsch 33:306-318.

Horzinek, M. C., P. S. van Wielink, and D. J. Ellens. 1975. Purification and electron microscopy of Lactic Dehydrogenase Virus of mice. J. Gen. Virol. 26:217-226.

Howard, C. J., M. C. Clarke, and J. Brownlie. 1985. An enzyme-linked immuno-sorbent assay (ELISA) for the detection of antibodies to bovine viral diarrhea virus (BVDV) in cattle sera. Vet. Microbiol. 10:359-369.

Howard, C. J., J. Brownlie, and L. H. Thomas. 1986. Prevalence of bovine virus diarrhoea virus in cattle in the U.K. Vet. Rec. 119:626-629.

Inaba, Y., T. Omori, and T. Kumagai. 1963. Detection and measurement of noncyto-pathogenic strains of virus diarrhea of cattle by the END method. Arch. ges Virusforsch 13:425-429.

Jerne, N. K. 1960. Immunological speculations. Ann. Rev. Microbiol. 14:341-358.

Johnson, D. W., and C. C. Muscoplat. 1973. Immunologic abnormalities in calves with chronic bovine viral diarrhea. Am. J. Vet. Res. 34:1139-1141.

Jubb, K. V. F., and P. C. Kennedy. 1985. Pathology of domestic animals. Vol. 2. P.C. Kennedy, and N. Palmer, eds. Academic Press, New York.

Juntti, N., B. Larson, and C. Fossum. 1987. The use of monoclonal antibodies in enzyme linked immunosorbent assays for detection of antibodies to bovine viral diarrhoea virus. J. Vet. Med. B34:356-363.

Justewicz, D. M., R. Magar, G. Marsolais, and J. Lecomte. 1987. Bovine viral diarrhea virus-infected MDBK monolayer as antigen in Enzyme-linked immuno-sorbent assay (ELISA) for the measurement of antibodies in bovine sera. Vet. Immun. Immunopath. 14:377-384.

Kahrs, R. F. 1981. Viral diseases of cattle. Iowa State Univ. Press, Ames, Iowa.

Katz, J. B., and S. K. Hanson. 1987. Competitive and blocking enzyme-linked immunoassay for detection of fetal bovine serum antibodies to bovine viral diarrhea virus. J. Virol. Meth. 15:167-175.

Kendrick, J. W. 1971. Bovine viral diarrhea-mucosal disease virus infection of pregnant cows. Am. J. Vet. Res. 32:533-544.

Ketelsen, A. T., D. W. Johnson, and C. C. Muscoplat. 1979. Depression of bovine monocyte chemotactic response by bovine viral diarrhea virus. Infect. and Immun. 25:565-568.

Kimura-Kuroda, J., and K.Yasui. 1983. Topographical analysis of antigenic determinants on envelope glycoprotein V3 (E) of Japanese encephalitis virus using monoclonal antibodies. J. Virol. 45:124-132.

Kimura-Kuroda, J., and K.Yasui. 1984. Antigenic comparison of envelope protein E between Japanese encephalitis virus and some other flaviviruses using monoclonal antibodies. J. Gen. Virol. 67:2663-2672.

Kniazeff, A. J., and W. P. Pritchard. 1960. Antigenic relationships in the Bovine Viral Diarrhea-Mucosal Disease complex. Proc. U.S. Livestock Sanit. Assoc. 64:344-350.

Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495-496.

Laude, H., and J. Guelfi. 1979. Properties of border disease virus as studied in a sheep cell line. Arch. Virol. 62:341-346.

Leavitt, R., S. Schlesinger, and S. Kornfeld. 1977. Tunicamycin inhibits glycosylation and multiplication of Sindbis and vesicular stomatitis virus. J. Virol. 21:375-385.

Lee, K. M., and J. H. Gillespie. 1957. Propagation of virus diarrhea virus of cattle in tissue culture. Am. J. Vet. Res. 18:952-953.

Liauw, H., and A. K. Eugster. 1986. Enzyme-linked immunosorbent assay for the diagnosis of bovine viral diarrhea. Southwest. Vet. 37:47-50.

Liess, B., H. R. Frey, and D. Prager. 1977. Antibody response of pigs following experimental infections with strains of hog cholera, bovine viral diarrhea. In: Hog Cholera - Classical Swine Fever and African Swine Fever. Com. Euro. Commun. Eur 5904, pp. 200-211.

Liess B., H. R. Frey, and S. Orban. 1983. Bovine virus diarrhea (BVD)-mucosal disease: persistent BVD field virus infections in serologically selected cattle. Dtsch. Tieraezth. Wochenschr. 90:249-296.

Liess, B., S. Orban, H. R. Frey, G. Trautwein, W. Wiefel, and H. Blindow. 1984. Studies on transplacental transmissibility of a bovine virus diarrhoea (BVD) vaccine virus in cattle. II. Inoculation of pregnant cows without detectable neutralizing antibodies to BVD virus 90-229 days before parturition (51st to 190th day of gestation). J. Vet. Med. B 31:669-681.

Lillie, L. E. 1974. The bovine respiratory disease complex. Can. Vet. J. 15:233-242.

Littlejohns, I. R., and K. H. Walker. 1985. Aetiology and pathogenesis of mucosal disease of cattle: current concepts, observations, and speculation. Aust. Vet. J. 62(3):101-103.

Lostrum, M. E., M.R. Stone, M. Tam, W. N. Burnette, A. Pinter, and R. C. Nowinski. 1979. Monoclonal antibodies against Murine Leukemia viruses: Identification of six antigenic determinants on the p15(E) and gp70 envelope proteins. Virology 98:336-350.

Maess, J., and E. Retzlo. 1970. Electron optical studies of bovine viral diarrhea-mucosal disease virus (BVDV). Arch. ges. Virusforsch. 30:39-46.

Magar, R., H. C. Minocha, and J. Lecomte. 1988. Bovine viral diarrhea virus proteins: Heterogeneity of cytopathogenic and non-cytopathogenic strains and evidence of a 53k Glycoprotein neutralization epitope. Vet. Microbiol. 16:303-314.

Malmquist, W. A. 1968. Bovine viral diarrhea-mucosal disease complex: etiology, pathogenesis, and applied immunity. J. Amer. Vet. Med. Assoc. 152:763-768.

Matthaeus, W. 1979. Detection of three polypeptides in preparations of bovine diarrhoea virus. Arch. Virol. 59:299-305.

Matthews, R. E. F. 1982. Classification and nomenclature of viruses. Mathews, ed. 4th Report of the International Committee on Taxonomy of Viruses. Karger Basel.

Mathews, J. M., and J.T. Roehrig. 1985. Determination of protective epitopes on the glycoprotein of Venezuelen equine encephalitis virus by passive transfer of monoclonal antibodies. J. Immunol. 129:2763-2767.

McClurkin, A. W, M. F. Coria, and R. C. Cutlip. 1979. Reproductive performance of apparently healthy cattle persistently infected with bovine viral diarrhea virus. Am. J. Vet. Res. 174:1116-1119.

McClurkin, A. W., E. T. Littledike, R. C. Cutlip, G. H. Frank, M. F. Coria, and S. R. Bolin. 1984. Production of cattle immunotolerant to bovine viral diarrhea virus. Can. J. Comp. Med. 48:156-161.

McClurkin, A. W., S. R. Bolin, and M. F. Coria. 1985. Isolation of cytopathic and noncytopathic bovine viral diarrhea virus from the spleen of cattle acutely and chronically affected with bovine viral diarrhea. J. Amer. Vet. Med. Assoc. 186:568-569.

Mengeling, W. L., D. E. Gutekunst, A. L. Fernelius, and E. C. Pirtle. 1963. Demonstration of an antigenic relationship between hog cholera and bovine viral diarrhea viruses by immuno-fluorescence. Can. J. Comp. Med. 27:162-164.

Meyling, A. 1984. Detection of BVD virus in viremic cattle by an indirect immunoperoxidase technique. Pp. 37-46. M. S. McNulty and J. B. McFerrin, eds. In Recent advances in viral diagnosis. Martinus Nijhoff, Boston.

Moennig, V. 1971. Untersuchingen uber das Sedimentations-verhalten der Ribonukleinsaure des Virus der Mucosal Diseas-Virusdiarrhoe: Ein Beitrag zu seiner Klassifizierung asl Mitglies der Togavirusgruppe. Thesis, Veterinary School Hannover.

Moennig, V., S. R., Bolin, A. Mateo, N. E. Kelso-Gourley, I. Wilke, and B. Liess. 1988. Identification of a single receptor for bovine viral diarrhea virus (BVDV) on the surface of cultured bovine cells. Abstr. Ann. Meet. Amer. Soc. for Microbiol. p. 326. Miami Beach, FL, 8-13 May 1988.

Moennig, V., A. Mateo, I. Greiser-Wilke, S. R. Bolin, N. E. Kelso, and B. Liess. 1989. A topological and functional epitope map of the surface of bovine viral diarrhea virus. Abstr. Ann. Meet. Amer. Soc. for Microbiol. p. 390. New Orleans, LA, 14-18 May 1989.

Muscoplat, C. C., D. W. Johnson, and J. B. Stevens. 1973a. Abnormalities of in vitro lymphocyte responses during bovine viral diarrhea virus infection. Am. J Vet. Med Res. 34:753-755.

Muscoplat, C. C., D. W. Johnson, and J. B. Stevens. 1973b. Abnormality in invitro lymphocyte responses during bovine viral diarrhea virus infection. Am. J. Vet. Res. 34:753-755.

Nakane, P. K., and G. B. Pierce. 1966. Enzyme-labeled antibodies: preparation and application for the localization of antigens. J. Histochem. Cytochem. 14:929-935.

Neukirch, M., B. Liess, H. R. Frey, and D. Prager. 1980. Serologische Beziehungen zwischen Stammen des Europaischen Schweinepest-Virus und dem Virus der Bovinen Virusdiarrhoe. Fortschritte der Verinarmed. 30:148-153.

Nuttall, P. A. 1980. Growth characteristics of two strains of bovine virus diarrhea virus. Arch. Virol. 66:365-369.

Nuttall, P. A., E. J. Stott, and L. H. Thomas. 1980. Experimental infection of calves with two strains of BVDV: virus recovery and clinical reactions. Res. Vet. Sci. 28:91-95.

Nystedt, H. 1960. Umea-sjukan. En Smittsan Virus beringad Slemhinnein-fektion hos Notkreatun: Vasterbotten Medlemsblad for Sveriges Veterinarforbund. 12:129-132

Olafson, P., and C. G. Rickard. 1947. Further observations on the virus diarrhea (new transmissible disease) of cattle. Cornell Vet. 37:104-106.

Olafson, P., A. D. MacCallum, and F. H. Fox. 1946. An apparently new transmissible disease of cattle. Cornell Vet. 36:205-213.

Orban, S., B. Liess, S, M. Hafez, H. R. Frey, H. Blindow, and B. Sasse-Patzer. 1983. Studies on transplacental transmissibility of a bovine virus diarrhoea (BVD) vaccine virus. I. Inoculation of pregnant cows 15 to 90 days before parturition (190th to 265th day of gestation). J. Vet. Med. B 30:619-634.

Padlan, E. A., E. W. Silverton, S. Sheriff, G. H. Cohen, S. J. Smith-Gill, and D. R. Davies. 1989. Structure of an antibody-antigen complex: crystal structure of the HyHEL-10 Fab-lysozyme complex. Proc. Nat. Acad. Sci. USA 86:5938-42.

Parks, J. B., R. F. Pritchett, Y. C. Zee. 1972. Buoyant density of bovine viral diarrhea virus (36511). Proc. Soc. Exp. Biol. Med. 140:595-598.

Peters, W., B. Liess, H. R. Frey, and G. Trautwein. 1987. Incidence of impact of persistent infection with BVD virus in the field. Pp. 133-143. J.W. Harkness, ed. In Pestivirus infections of ruminants. Office for official publications of the European Communities, Luxemburg,

Pizer, L. I., G. H. Cohen, and R. J. Eisenberg. 1980. Effect of tunicamycin on herpes simplex virus glycoprotein and infectious virus production. J. Virol. 34:142-153.

Pocock, D. H., C. J. Howard, M. C. Clarke, and J. Brownlie. 1987. Variation on the intracellular polypeptide profiles from different isolates of bovine virus diarrhea virus. Arch. Virol. 94:43-53.

Potgeiter, L. N., M. D. McCraken, F. M. Hopkins, R. D. Walker, and J. S. Guy. 1984. Experimental production of bovine respiratory tract disease with bovine viral diarrhea virus. Am. J. Vet. Res. 45:1582-1585.

Pritchard, W. R. 1961. The virus diarrhea - mucosal disease complex. Vet. Scope 6:2-16.

Pritchard, W. R. 1963. The bovine viral diarrhea-mucosal disease complex. Adv. Vet. Sci. 8:1-47.

Pritchard, W. R., D. B. Taylor, H. E. Moses, and L. P. Doyle. 1954. A virus diarrhea in cattle. Annual Report. Department of Veterinary Science, Ind. Agric. Exp. Stn. Project No. 724

Pritchard, W. R., D. B. Taylor, H. E. Moses, and L. P. Doyle. 1956. A transmissible disease affecting the mucosal of cattle. J. Am. Vet. Med. Assoc. 128:1-5.

Pritchett, R. F., and Y. C. Zee. 1975. Structural proteins of bovine viral diarrhea virus. Am. J. Vet. Res. 36:1731-1734.

Purchio, A. F., R. Larson, and M. S. Collett. 1983. Characterization of virus-specific RNA synthesized in bovine cells infected with bovine viral diarrhea virus. J. Virol. 48:320-324.

Purchio, A. F., R. Larson, and M. S. Collett. 1984. Characterization of bovine viral diarrhea proteins. J. Virol. 50:666-669.

Pustell, J., and F. C. Kafatos. 1982. A high speed high capacity homology matrix: zooming through SV40 and polyoma . Nucleic Acids Res. 10:4765-4782.

Pustell, J., and F. C. Kafatos. 1984. A conventional and adaptable package of computer programs for DNA and protein sequence management, analysis, and homology determination. Nucleic Acids Res. 12:643-655.

Radostits, O. M., and J. A. Henderson. 1983. Pp. 754-761. Diseases of cattle. In D. C. Blood, O. M. Radostits, and J. A. Henderson eds. Veterinary Medicine 6th ed. Bailliere Tindall, London.

Ramsey, F. K., and W. H. Chivers. 1953. Mucosal disease of cattle. North Am. Vet. 34:629-633.

Ramsey, F. K. 1954. The pathology of a mucosal disease. Am. Vet. Med. Assoc. Proc. 1954:162-166.

Reggiardo, C., and M. L. Kaeberle. 1981. Detection of bacteremia in cattle inoculated with bovine viral diarrhea virus. Am. J. Vet. Res. 42:218-221.

Renard, A., C. Guiot, D. Schmetz, L. Dagenais, P. P. Pastoret, D. Dina, and J. A. Martial. 1985. Molecular cloning of bovine viral diarrhea virus sequences. DNA 4:635-640.

Renard, A., D. Dina, and J. Martial. 1987. Vaccines and diagnostics derived from bovine diarrhea virus. European Patent Application number 86870095.6. Publication number 0208672, 14 January.

Revell, S. G., D. Chasey, T. W. Drew, and S. Edwards. 1988. Some observations on the semen of bulls persistently infected with bovine virus diarrhea virus. Vet. Rec. 123:122-125.

Roeder, P. L., and J. W. Harkness. 1986. BVD virus infection: prospects for control. Vet. Rec. 118:143-147.

Roehrig, J. J., J. A. Corser, and M. J. S. Schlessinger. 1980. Isolation and characterization of hybrid lines producing monoclonal antibodies directed against the structural proteins of Sindbis virus. Virology 101:41-49.

Roehrig, J. J., J. T. Day, and R. M. Kinney. 1982. Antigenic analysis of the surface glycoprotein of a Venezuelen equine encephalitis virus (TC-83) using monoclonal antibodies. Virology 118:269-278.

Roehrig, J. J., and J. H. Mathews. 1985. The neutralization site on the E2 glycoprotein of Venezuelen equine encephalitis (TC-83) virus is composed of multiple conformationally stable epitopes. Virology 142:347-356.

Romvary, J. 1965. Incidence of virus diarrhoea. Acta Vet. Hung. 15:451-455.

Roth, J. A., M. L. Kaeberle, and R. W. Griffith. 1981. Effects of bovine viral diarrhea virus infection on bovine polymorphonuclear leukocyte function. Am. J. Vet. Res. 42:244-250.

Roth, J. A., S. R. Bolin, and D. E. Frank. 1986. Lymphocyte blastogenesis and neutrophil function with bovine viral diarrhea virus. Am. J. Vet. Res. 47:1139-1141.

Ruitenberg, E. J., P. A. Steerenberg, and B.J. M. Brosi. 1975. Micro-system for the application of (ELISA) in the serodiagnosis of Trichinella spiralis infections. Medikon Nederland. 3:30-35.

Sabara, M., L. A. Babiuk, J. Gilchrist, and V. Misra. 1982. Effect of tunicamycin on Rotavirus assembly and infectivity. J. Virol. 43:1082-90.

Saravis, C. A. 1984. Improved blocking of nonspecific antibody binding sites on nitrocellulose membranes. Electrophoresis 5:54-55.

Schlesinger, J. J., E. E. Walsh, and M. W. Brandriss. 1984. Analysis of 17D Yellow fever virus protein epitopes using monoclonal antibodies. J. Gen.Virol. 65:1637-1644.

Schmaljohn, A. L., K. M. Kokubum, and G. A. Cole. 1983. Protective monoclonal antibodies define maturational and pH dependent antigenic changes in Sindbis virus E1 glycoprotein. Virology 130:144-154.

Sela, M. 1969. The nature of antigenic structures. Science 166:1365-1367.

Sheffy, B. E., L. Coggins, and J. A. Baker. 1962. Relationship between Hog cholera virus and virus diarrhea virus of cattle. Proc. Soc. Exp. Biol. Med. 109:349-352.

Smith, T. J., W. E. Brandt, J. L.Swanson, J. M. McCown, and E. L. Buescher. 1970. Physical and Biological properties of Dengue-2 virus and associated antigens. J. Virol. 5:524-532.

Snowdon, W. A., and E. L. French. 1968. The bovine mucosal disease-swine fever virus complex in pigs. Aust. Vet. J. 44:179-184.

Spiro, R. G. 1962. Studies on the monosaccharide sequence of the serum glycoprotein Fetuin. J. Biol. Chem. 237:646-652.

Steck, F. 1980. Immune Responsiveness in Cattle Fatally Affected by Bovine Virus Diarrhea-Mucosal Disease. Zbl. Vet. Med. 27:429-445.

Stober, M. 1984. Current knowledge of the BVD syndrome of cattle: agent, immune response, course and spread, control. Bov. Pract. 19:49-60.

Stott, E. J., J. D. Almeida, and K. J. O'Reilly. 1974. Characterization of mucosal disease as a togavirus by electron microscopy. Microbios. 11A:79-83.

Stott, E. J., L. H. Thomas, A. P. Collins, N. J. Jebett, G. S. Smith, P. D. Luther and R. Caswell. 1980. A survey of virus infection of the respiratory tract of cattle and their association with disease. J. Hyg. 85:257-270.

Strauss, E. G., and J. H. Strauss. 1977. Togaviruses. Pp. 111-166. D. P. Nayak ed. In The molecular biology of animal viruses Vol. I. Plenum Press, New York.

Thomas, R. G., and M. Savan. 1963. Studies on virus diarrhea and mucosal disease of cattle. Can. J. Comp.Med. 27:207-214.

Tulip, W. R., J. N. Varghese, R. G. Webster, G. M. Air, W. G. Laver, and P. M. Colman. 1989. Crystal structure of neuraminidase-antibody complexes. Cold Spring Harb. Symp. Quant. Biol. Pt. 1:257-263.

Tyler, D. E., and F. K. Ramsey. 1965. Comparative pathologic, immunologic, and clinical responses produced by selected agents of the bovine mucosal disease-virus diarrhea complex. Am. J. Vet. Res. 26:903-913.

Underdahl, N. R., O. D. Grace, and A. B. Hoerlein. 1957. Cultivation in tissue culture of cytopathogenic agent from bovine mucosal disease. Proc. Soc. Biol. and Med. 94:795-799.

Vantsis, J. T., R. M. Barlow, J. Fraser, J. C. Rennie, and D. L. Mould. 1976. Experiment in Border disease VIII. Propagation and properties of a cytopathic virus. J. Comp. Pathol. 86:111-120.

Voller, A., D. E. Bidwell, G. Huldt, and E. Engvall. 1974. A microplate method of enzyme-linked immunosorbent assay and its application to malaria. Bull. World Health Org. 51:209-211.

Voller, A., and D. E. Bidwell. 1975. A simple method for detecting antibodies to Rubella. Brit. J. Exp. Pathol. 56:338-344.

Voller, A., and D. E. Bidwell. 1976. Enzyme-immunoassays for antibodies in measles, cytomegalovirus infections, and after rubella vaccination. Brit. J. Exp. Pathol. 57:243-247.

Webster, R., and G. Laver. 1980. Determination of the number of nonoverlapping antigenic areas on Hong Kong (H3N2) Influenza virus hemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. Virology 104:139-148.

Westaway, E. G., M. A. Brinton, S. Giadamovich, M. C. Horzinek, A. Igarashi, L. Kaariainen, D. K. Lvov, J. S. Porterfield, and D. W. Trent. 1985. Togaviridae: Intervirology 24:125-139.

Westenbrink, F., W. G. J. Middel, P. J. Straver, and P. W. de Leeuw. 1986. A blocking Enzyme-Linked Immunosorbent Assay (ELISA) for Bovine Virus Diarrhoea Virus Serology. J. Vet. Med. B33:354-361.

Whitmore, H. L., B. K. Gustafsson, P. Havareshti, A. B. Duchateau, and E. C. Mather. 1978. Inoculation of bulls with bovine virus diarrhea virus: excretion of virus in semen and effects on semen quality. Theriogenology 9:153-169.

Wincker, R., and S. Avrameas. 1969. Localization of virus antigens by enzyme-linked antibodies. J. Gen. Virol. 4:465-472.

Woods, G. T., M. E. Mansfield, and G. F. Cmarik. 1973. Effects of bovine virus diarrhea and parainfluenza-3 virus vaccines on development of respiratory tract disease in calves. J. Am. Vet. Med. Assoc. 163:743-748.

Yates, W. D. G. 1982. A review of infectious bovine rhinotracheitis, shipping fever pneumonia, and viral-bacterial synergism in respiratory disease of cattle. Can. J. Comp. Med. 46:225-263.

Yewdell, J. W., and W. Gerhard. 1981. Antigenic characterization of viruses by monoclonal antibodies. Ann. Rev. Microbiol. 35:185-206.

Zeegers, J. J. W., and M. C. Horzinek. 1977. Pp. 52-57. In "Hog Cholera/Classical Swine Fever and African Swine Fever" Com. Euro. Comm. Assoc. EUR 5904.

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